THE UNIVERSITY
of EDINBURGH

THE UNIVERSITY of EDINBURGH

Determining the role of androgen receptor and glucocorticoid receptor in the rodent adrenal cortex through conditional gene targeting

Anne-Louise Gannon

MRes, University of Dundee

BSc, University of Dundee

Medical Research Council
Centre for Reproductive Health
The Queen's Medical Research Institute
47 Little France Crescent
Edinburgh, EH16, 4TJ

Thesis submitted to the University of Edinburgh for the degree of Doctor of Philosophy

September 2017

Declaration

I hereby declare that the experimental work described in this thesis, is the sole work of the author. Results obtained through collaboration with or completed by other individuals and institutions are appropriately indicated and acknowledged. These studies have not been submitted in supporting another degree or qualification at the University of Edinburgh, or any other institute.

Anne-Louise Gannon

September 2017

Contents

Table of Figures

Table of Tables

Acknowledgements

I cannot describe the relief and excitement to be finally writing this section of my thesis, and I definitely could not have got to this point without help and support from a fantastic group of people.

First and foremost I would like to give a massive thanks to my supervisor, Professor Lee Smith. Thank you for giving me the opportunity to come and be part of such an amazing lab, for seeing potential in me, even though I was definitely not qualified for the position! Thank you for giving me the opportunity to learn and develop as a scientist, for providing never ending support, encouragement, advice, and plenty of WTFs! I would definitely have not got to this stage without all of this, and will always be grateful.

I would like to thank my supervisors, Rod Mitchell and Ian Mason, for helpful advice, encouragement and laughs during meetings and throughout my project.

I would also like to give a massive thank you to Laura O'Hara. Throughout my whole PhD you have encouraged me and provided me with an abundance of support. Thank you for challenging me, finding holes in my logic, pushing me to continually improve and for being a massive source of strength for me in these final stages. I hope my bread mice and chai latte tests have not bamboozled you too much!

Thank you to the amazing Smith group, past and present for all the help, advice and support. Throughout the course of my PhD I have not only had great colleagues in the Smith group but have made amazing friendships, so thank you to Annalucía and Diane for lots of laughs and adventures inside and outside work and thank you to Mike for numerous chats and laughs throughout our writing process. I definitely would not have gotten as far without lots of lab training and technical support from Laura Milne, Sarah, Hazel, Rosa and Nathan, who have all assisted me in animal work, histology and serum hormone analysis. Thank you all for teaching me so much and never losing patience, in particular Nathan, who definitely bore the brunt of my nonsense! I honestly can't thank you all enough.

Thank you to my wonderful friends Pandora, Katie and Naomi who have been amazing support throughout my eight years of university and for sticking me this long!

I would also like to thank my amazing family for all their support, love and encouragement, who always pushed me to continue and better myself. You have picked me up and pushed me on, on more than one occasion. I hope this makes you all proud! I would also like to thank my amazing partner in crime David. You have loved me at my best and you have loved me at my worst, and I can't thank you enough for your unwavering support, encouragement and for all our adventures throughout my PhD, this would not have been possible without you.

Abstract

Androgens are well documented as important regulators of male health, primarily in the maintenance and development of male sexual characteristics. However, a decline in circulating androgens has also been associated with co-morbidities such as obesity, cardiac disease and metabolic syndrome. Previous research has focussed upon the body wide impact of adrenal androgens, however whilst androgen receptor (AR) is abundantly expressed in the adrenal cortex of both rodents and humans, surprisingly little is known about androgen action on the adrenal cortex itself. This gap in our understanding is at least in part due to the perceived lack of suitable animal models. Rodents have largely been overlooked as a model system as their adrenals are unable to produce androgens due to lack of 17α Hydroxylase and 17, 20 lyse activity and they therefore do not have a zona reticularis. However, historical studies using castrated mice showed that removal of androgens leads to the redevelopment of an additional cortex zone known as the transient X-zone. The foetal adrenal is thought to give rise the adult adrenal cortex in human and rodents. These foetal cells are maintained for a period postnatally and regress differently depending on species and sex. In the human this zone is known as the 'foetal zone', and the rodent homologue termed the 'X-zone'. The mechanisms underpinning the regression of the X-zone and its purpose and maintenance postnatally still aren't clearly understood.

To provide a comprehensive overview of androgen signalling in the adrenal cortex, multiple mouse models were utilised. First, Cre/*loxP* technology was used to ablate AR specifically from the adrenal cortex. Further androgen manipulation was achieved through castration (removal of androgens) and human chorionic gonadotropin (hCG) treatment (increased androgens). The initial study investigates the impacts on the male mouse adrenal. Histology analysis revealed the presence of an X-zone in all experimental cohorts following loss of AR or circulating androgens, confirmed by 20-α-hydroxysteroid dehydrogenase (20 alpha-HSD) expression. These data demonstrate that androgens signalling via AR is required for X-zone regression during puberty. However, interrogation of morphology of hCG treated cohorts revealed no phenotypic changes compared to controls, this demonstrates that hyper stimulation with androgens does not negatively impact the adrenal cortex or influence X-zone morphology.

Differences in X-zone morphology and 20 alpha-HSD localization prompted cortex measurements which revealed significant differences in X-zone depth and cell density depending on ablation of AR, circulating androgens or both. This suggests that androgens and androgen receptor are working together and also independently to regulate the adrenal cortex. This result was strengthened through analysis of steroid enzyme genes and cortex markers, which revealed that normal AKR1B7 expression was absent following loss of androgens but not androgen receptor. A final part of this study examined the impacts long term androgen receptor ablation and long term castration in ageing animals.

A final part of this study examined the impacts long term androgen receptor ablation and long term castration in ageing animals. These results demonstrate that following prolonged loss of androgens that there is no major disruption to the adrenal cortex. Morphology analysis and X-zone measurements revealed that X-zone regression was occurring in mice with long term castration, characterized by a reduction in size and pockets of vacuolization throughout the X-zone. This phenotype is also observed in ageing females with X-zone regression via vacuolization. These data suggest that following prolonged loss of androgens, the male adrenal is feminized and behaves as such. In contrast, AR ablation only, results in an enlarged adrenal with large spindle cell lesions and X-zone expansion confirmed by X-zone measurements. Initial experiments have demonstrated that androgens can work independently of AR to regulate the adrenal cortex. Together these data suggests that AR is required to control the appropriate action of circulating androgens in the adrenal cortex, with loss of AR resulting in off target signalling from circulating androgens in the adrenal leading to spindle cell hyperplasia, X-zone expansion and X-zone mislocation.

A second set of studies were carried out to determine the role of androgen signalling in the female adrenal, specifically, if loss of AR leads to the absence of normal X-zone regression during pregnancy. To answer this question the same selective AR ablation model was used. Analysis of litters comparing observed and expected genetic distribution revealed significantly fewer females being born carrying complete ablation of adrenal AR. Morphology analysis of these mice revealed severe cortex disruption and spindle cell hyperplasia similar to that observed in mutant males. Investigation of adrenals following pregnancy revealed that X-zone regression still

occurred despite loss of AR. This result shows that X-zone regression in the female is under different regulation compared to male adrenal and occurs via an androgen-independent signalling mechanism. However, loss of AR still leads to anatomical dysregulation of the adrenal cortex.

AR ablation revealed changes in glucocorticoid receptor (GR) expression in the adrenal cortex. To dissect this relationship further a final study was conducted, attempting to ablate GR from the adrenal cortex also using the *Cyp11a1* Cre. Initial observations of these mice revealed excessive hair loss through barbering, curved spines and stressed behaviour when monitored in the cage under normal conditions. Immunohistochemistry was used to confirm GR ablation in the adrenal cortex, however, to our surprise, GR expressing cells were not steroidogenic and thus were not targeted by the Cre recombinase. Despite no GR ablation in the adrenal, morphology analysis revealed severe disruption to the adrenal cortex. The *Cyp11a1* Cre not only targets the adrenal but is expressed in the hindbrain. To determine if GR ablation in the hindbrain explains the phenotype, we next used PCR analysis interrogating hindbrain genomic DNA to determine if there was recombination of GR. Results confirmed GR recombination in the hindbrain. Due to the observation of stressed behaviour and adrenal cortex disruption, we wanted to determine if this was a result of hyperactivity of the adrenal cortex. Serum corticosterone was analysed and was elevated in these animals. These data revealed that GR ablation in the hindbrain results in adrenal cortex disruption and an elevated stress response, potentially highlighting a new model to investigate stress disorders and their impact on the hypothalamic-pituitary-adrenal axis.

Together this data defines new roles for AR signalling in the adrenal cortex and the role of the hindbrain GR signalling in regulating adrenal morphology and function.

Lay Summary

The adrenal glands are small glands located on top of each kidney. They produce hormones such as adrenaline and stress hormones such as cortisol, they also produce sex steroids known as androgens. Androgens are well documented as an important regulator of male health, primarily in the maintenance and development of male sexual characteristics. A decline in circulating testosterone has also been associated with a number of conditions such as obesity, cardiovascular disease and metabolic syndrome. Previous research has focussed upon the body-wide impact of adrenal testosterone although surprisingly little is known about testosterone regulation on the adrenal itself. This gap in our understanding is at least in part due to the perceived lack of suitable animal models. Rodents have largely been overlooked as a model system as their adrenals are unable to produce androgens but historical studies using castrated mice showed that removal of androgens leads to the redevelopment of an additional cell population in the adrenal known as the X-zone, suggesting the adrenal does respond to androgens. The fetal adrenal is thought to provide the cells needed to develop the adult adrenal in both human and rodents. These fetal cells are maintained for a short period after birth and are removed differently depending on species and sex. In the human these cells are known as the 'fetal zone', and the rodent complement termed the 'X-zone'. The mechanisms controlling the removal of the X-zone and its purpose and maintenance after birth still aren't clearly understood.

This thesis is comprised of three main studies. The first study uses a mouse model that is able to remove testosterone's receptor known as androgen receptor or 'AR' from the adrenal. Additional castrated models were used to remove androgens circulating round the body. This permitted the examination of the impacts of losing AR, androgens or both within the male adrenal. Results revealed the presence of X-zone cells in the adrenal that should have regressed during puberty in males. This tells us that androgens need AR to be able to remove these cells. X-zone cells were observed in all mouse models following removal of AR or testosterone. Investigation of the structure of these X-zone cells revealed differences in size and density depending on whether AR or testosterone had been removed, suggesting testosterone can also work independently of AR. A final experiment set out to examine the impacts of long term loss of

testosterone signalling. This revealed that removal of androgens results in no further damage to the adrenal but prolonged loss of AR resulted in severe adrenal disruption and large lesions known as spindle cell hyperplasia developed. This highlighted that AR is needed to control the effects of androgens on the adrenal.

A second study to investigate the role of androgen signalling in the female adrenal was conducted using the same mouse model. Results describe the same spindle cell lesions observed in the aged male adrenal, however the X-zone cells that usually regress during pregnancy in females still regressed as normal. This showed that X-zone cells in the female are not controlled by AR or testosterone. Despite this, loss of AR still leads to damage to the adrenal.

The final study aimed to investigate the relationship between the adrenal and a receptor responsible for stress response known as glucocorticoid receptor or 'GR'. Using a mouse model we attempted to remove GR from the adrenal, however, this model resulted in removal of GR from the hindbrain instead. This disruption to GR resulted in mice that display stress and anxiety behaviors. These stress behaviors were shown through excess hair removal known as barbering and persistent digging in one place with no exploration around the cage. Investigation of the adrenal revealed large amounts of damage cell death. This study highlights the impacts of hindbrain GR and its role in regulating the adrenal. In addition to this, it potentially provides a new model to be able to investigate the development of stress disorders.

In summary, the results of these studies have increased our understanding of the complex relationship between AR and androgen signalling in the adrenal. Additionally, we have defined new roles for hindbrain GR regulation of the adrenal, and a potential new mouse model for investigation of stress disorders.

Presentations relating to this thesis

1) Poster presentation at the 19th European Congress of Endocrinology, Lisbon, 2017. **Androgen receptor signalling is essential for regression of the x-zone and regulation of the adrenal cortex in the male mouse.**

2) Oral poster presentation at the 18th Adrenal cortex conference, Boston. 2016. **The role of androgens in the development and function of the male adrenal gland in the mouse.**

Chapter 1: Literature Review

1.1 The Adrenal Gland

The adrenal glands are a pair of small endocrine organs located on top of each kidney. Each gland comprises of essentially two different organs, the outer adrenal cortex and the inner adrenal medulla. The adrenal cortex is further divided into concentric zones. In humans these zones are known as the zona glomerulosa (ZG), zona fasciculata (ZF), and the zona reticularis (ZR) (1). The adrenal cortex is a dynamic tissue which possess the capability to renew its zones by replacing senescent cells with newly differentiated ones (2). This ability to renew its cortex zones permits organ remodelling to appropriately deal with the physiological demands for steroids. Each zone of the adrenal is responsible for the production of specific steroids (3). The adrenal medulla is located in the centre of the adrenal cortex, which also plays a role in immediate stress response (4).

1.1.2 Adrenal Cortex Histology

The zona glomerulosa is the outermost cortex zone found directly under the adrenal capsule. The cells that make up the ZG are ovoid in shape and are arranged in clusters or arches, these cells are separated by trabeculae that are continuous with the capsule (5). These cells are important for the production and secretion of the hormone aldosterone responsible for sodium balance and water reabsorption in the kidney (6). The zona fasciculata is the middle and largest zone of the adrenal cortex, comprised of cells organised into bundles or fascicles (7). The ZF is described as being columnar in appearance and is the primary producer of glucocorticoid hormones responsible for stress response (8). The final and innermost zone of the cortex in known as the zona reticularis. The cells in this zone are arranged in cord like structures, these cords project in different directions giving the appearance of a ‘net’ structure. These cells are responsible for the production of androgens (9). The zones that are present can vary between species. To frame the parameters for this thesis, adrenal formation in the

mouse is also described, which only present with a zona glomerulosa and zona fasciculata (10).

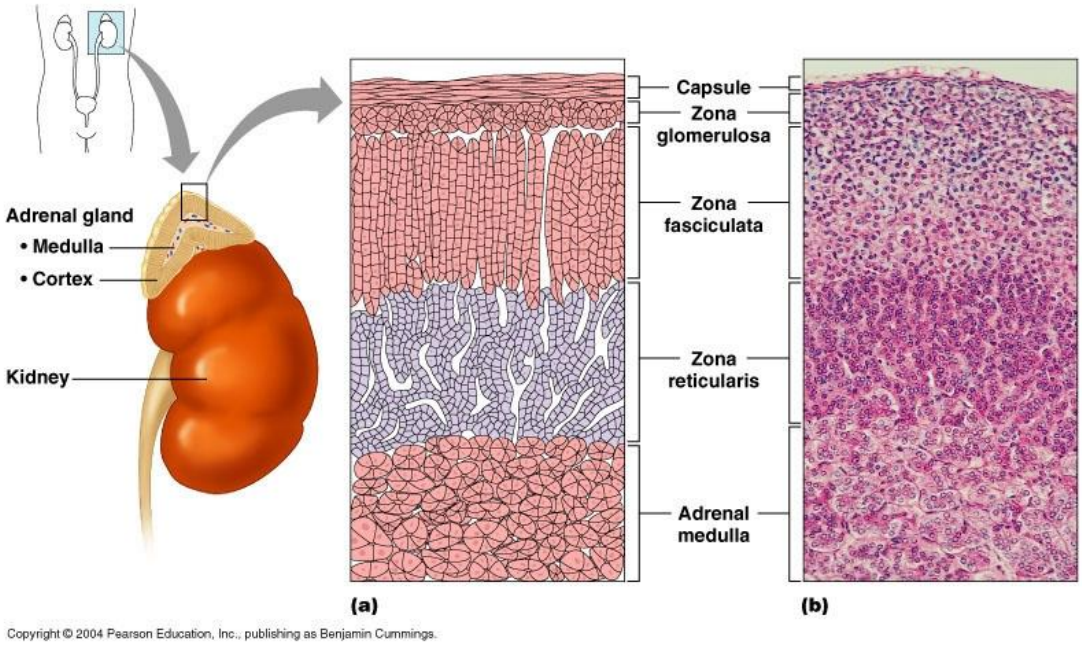


Figure 1-1. Location and histology of the adrenal glands. *Schematic detailing the location of the adrenal and its cortex zones. (A) Diagram describing the shape and location of the different zones in the adrenal cortex. (B) H&E image comparing diagram and human adrenal zones. Figure adapted from (11).*

1.1.3 Cortex maintenance and renewal

The adrenal plays an important role in many physiological processes; to be effective in doing so it needs to tightly regulate and renew its zones to deal with the changing demand for steroid production. Cortex zones have the ability to reversibly expand, contract and alter biochemical processes to accommodate needs and maintain homeostasis. The adrenal cortex is described as a dynamic tissue, this is achieved through the replacement of senescent cells with newly differentiated cells and appropriate clearance of injured or old cells. The mechanisms in which the adrenal regulates its zones was first summarized by Jones in 1957 (12), it was proposed that cell renewal occurs through inward migration of new cells from the periphery of the cortex to the cortex/medulla boundary and that other cells should then undergo

clearance to permit this dynamic system. This theory was later confirmed through multiple experiments that demonstrated the presence of a progenitor cell population in the sub-capsular region of the adrenal, in which these cells are able to give rise to the differentiated cells that populate the cortex. This was initially demonstrated through following of labelled cells after the pulse administration of titrated thymidine (13, 14). This was more recently demonstrated via bromodeoxyuridine and [^{3}H]thymidine labelling to provide lineage tracing of cells as they migrate through the cortex (15). One of the most noted studies that clearly demonstrated the regenerative capability of the adrenal cortex was performed in the rat adrenal and involved the enucleation of the adrenal cortex and medulla leaving only capsule. Following this removal, the adrenal cortex was repopulated in six weeks (16). This finding was also backed by additional studies carried out in bovine adrenocortical cells that were transplanted under kidney capsule in immunocompromised mice, and these cells gave rise to adrenocortical tissue capable of producing steroids (17, 18). Additionally, it has also now been demonstrated that cells do in fact move centripetally until they reach the cortex boundary and become apoptotic (15, 19).

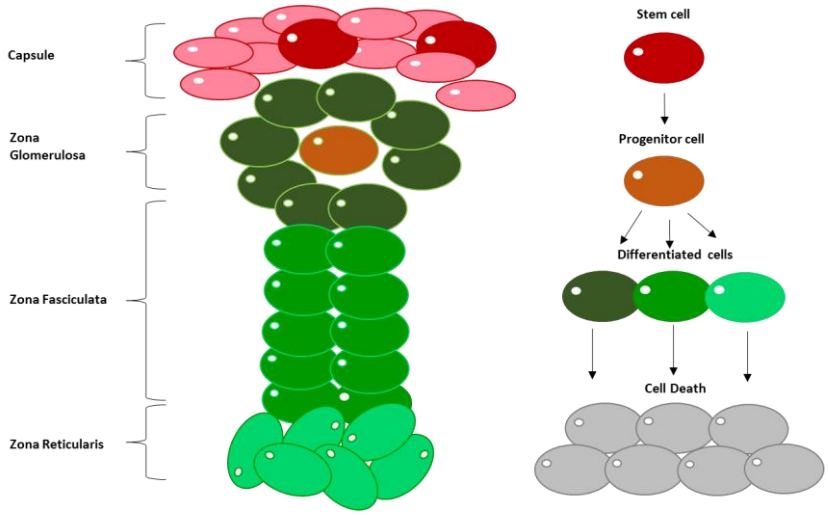


Figure 1-2. The cortex is populated from the capsule to the cortex medulla boundary. *Diagram depicting the mode of action of cell migration and population of the adrenal cortex. Stem cells located in the in the sub capsule region give rise to progenitor cells located in the glomerulosa. These stem and progenitor cells differentiate to populate to the ZF, ZG and ZR.*

1.1.3.1 Adrenocortical stem cells

Additional studies have investigated the processes of cortex renewal and have revealed that the regulation of zonation is much more complex than the initial paradigm described. Much research has gone into identifying locations throughout the cortex that may contain stem and progenitor cells. They identified the capsule, subjacent cortex and the juxtamedullary region as also containing stem and progenitor cells (20). These studies also highlighted that they were important through different time points and required for responses to extreme physiological stress. Although not the normal mechanisms, under certain conditions the adrenal has been shown to be able to populate the cortex via centrifugal migration (20). This was demonstrated following gonadectomy, which resulted in the development of a cortex zone termed the X-zone (21, 22) adjacent to the medulla, (discussed in depth in section 1.5.3), which subsequently populated the adrenal cortex from a pool of foetal stem cells. These foetal cells also termed X-zone cells, differ from the stem/progenitor populations located in the capsule and sub capsule region. They have been shown to have differences in the structure of their mitochondrial cristae and stain for their own independent marker, 20alpha-hydroxysteroid dehydrogenase (23, 24). The mechanisms controlling this cell population and their function are still not clearly understood. A list of the known progenitor cells can be seen in table 1-1.

Stem/Progenitor Population	**Location**	**Reference**
$WT1^+$	Capsule	(25)
$GLI1^+$	Capsule	(26-28)
$TCF21^+$	Capsule	(26)
SHH^+	Subcapsular Region	(26-28)
Foetal adrenal	Juxtamedullary region	(21, 29, 30)

Table 1-1. Table describing Stem/progenitor populations in the adrenal cortex.

1.1.3.2 Triggers for cortex remodelling

Various experiments have detailed the changes in size of cortex zones under certain biological processes or treatments. The zona glomerulosa has been shown to expand upon a decrease in sodium or an increase potassium induced through diet (5, 31), and contract the zone and decrease steroid production with the inverse. Adrenocorticotropic hormone (ACTH) has been shown to cause expansion of the zona fasciculata with a subsequent increase in steroid production, consequently treatment with a corticosteroid medication dexamethasone has the opposite effect (32, 33).

Psychological pressures have also been seen to induce remodelling, an interesting study examining social status in the marmoset revealed functional changes in the zona reticularis. The rodent X-zone remodelling has been shown to be impacted by a number of processes, through development, puberty and pregnancy and showing sexually dimorphic regulation of this zone (34-40). The cortex remodelling that occurs due to the aforementioned processes are not to be confused with adrenal hyperplasia. The expansion and reduction of adrenal zones is essential to cope with demands for certain steroids. However, adrenal hyperplasia, which results from overstimulation or inherited genetic conditions, disrupts steroid biosynthesis in the adrenal which results in inappropriate enlargement of the adrenal cortex (22, 41, 42).

Chapter 1: Literature Review

1.2 Signalling pathways involved in cortex maintenance and remodelling

An abundance of research has been conducted to identify pathways involved in cortex maintenance and function. Key pathways with particular importance to adrenal cortex remodelling are described in detail in subsequent sections (1.3.1-1.4.4). However, are summarised in table 1-2.

Pathways	**Reference**
Hedgehog Pathway	(28, 43, 44)
Fibroblast growth factor pathway	(45)
WNT/ β-Catenin pathway	(30, 46-48)
cAMP Signalling	(29, 49)
IGF Signalling	(50, 51)
Transforming growth factor β signalling	(52-54)
LH Signalling	(55, 56)
Transcription factors	**Reference**
SF1	(57, 58)
DAX1	(59-61)
WT1	(25, 62)
GATA-6	(63-65)

Table 1-2. Pathways and transcription factors involved in cortex remodelling.

1.2.1 Hedgehog pathway

The hedgehog pathway is essential in regulating aspects of tissue maintenance, renewal and regeneration. The hedgehog family is comprised of a family of morphogens named sonic hedgehog (*Shh*), Indian hedgehog (*Ihh*) and desert hedgehog (*Dhh*) (66). The only member of this family present in the mouse adrenal gland is SHH and can be detected early in foetal development, and becomes restricted to a specific cell population in the outer ZG in adulthood. SHH exerts its effects via the Patched-1 (PTCH1) receptor, a transmembrane receptor found on target cells. PTCH1 inhibits

the G-protein coupled receptor Smoothened (SMO) in the absence of SHH. SMO signalling promotes the nuclear localisation of *Gli1* transcription factors. Binding of SHH to PTCH1 relieves the inhibition exerted on SMO and thus prevents proteolytic processing of GLI factors and allows them to act on their target genes (67). Transcriptional GLI1 is not expressed in the absence of SHH, however, it is upregulated by activation of this pathway. Therefore, *Gli1* expression can be a useful marker for active SHH signalling. Subcapsular adrenal cells that express *Sf1* secrete SHH, but do not secrete the terminal enzymes that permit the synthesis of corticoids (27). Cells that do not express *Sf1* can respond to SHH by expressing *Gli1*. Experiments examining cortical growth in embryo, foetus and new-born mouse highlighted that $GL1^+$ progenitor cells contribute to cortex formation. GLI^+ cells have been shown to migrate centripetally into the cortex, where they subsequently lose responsiveness to SHH and become steroidogenic, shown through the expression of *Cyp11b2* as a marker for ZG cells and *Cyp11b1* for ZF. Mouse models that conditionally ablate *Shh* from the adrenal demonstrated a shrunken adrenal cortex and capsule thinning. These results demonstrate that *Shh* signalling is required for normal cortex formation but not essential in zonation (27, 28).

1.2.2 WNT/β-Catenin pathway

The mammalian wingless-type MMTV integration site (Wnt) pathway has been shown to be integral in organogenesis, homeostasis, and stem cell biology. β-Catenin is able to control the interaction between cadherin and adheren complexes and also participate in canonical WNT signalling. For the purposes of WNT signalling, β-Catenin acts as a co-activator for the TCF/LEF family of transcription factors. Once transcriptionally active, detection of β-Catenin can be observed in the adrenal gonadal primordium (AGP), the adrenal primordium and adrenal subcapsular cells. WNT/β-Catenin signalling is thought to be important in the maintenance of undifferentiated adrenocortical/progenitor cells (68). Experiments which have examined $SF1^+$ positive cells with targeted mutagenesis of β-Catenin result in eventual adrenal hypoplasia (46). This is thought to be due to the eventual depletion of the stem progenitor pool,

ultimately resulting in adrenal failure. The complexity of the WNT/β-Catenin pathway allows for fine tuning in response to morphogen gradients, however, pathways with the ability to regulate cell self-renewal often result in the driving mechanism behind oncogenesis. The WNT/ β-Catenin pathway has been described as one of the most frequently mutated pathways in adrenocortical carcinomas (69).

1.2.3 cAMP pathway

Hormones involved in regulating proliferation of the adrenal cortex often work via binding to G-protein coupled receptors on the cell surface. Activating these receptors initiates the stimulation of adenylate cyclase, resulting in cyclic adenosine monophosphate (cAMP) production. Once activated, cAMP binds to its regulatory subunits of protein kinase A (PKA) subsequently permitting the phosphorylation of downstream targets, including those that can enhance expression of steroidogenic genes (49). Experiments examining cell specific ablation of the protein kinase-A regulatory subunit gene (*Prkar1a*) under an *Akr1b7* promoter results in excessive cAMP production in the adrenal cortex. This study noted that these mice when aged presented with symptoms consistent with the Carney complex and pituitary independent Cushing's syndrome. The study suggested that the development of these diseases was predicated on the resistance to apoptosis and increased proliferation. This study also demonstrated the development of an X-zone that was able to produce cortisol. These results highlighted the importance of PKA activity in the maintenance of the adrenal cortex (29).

1.2.4 LH signalling

Luteinising hormone (LH) is secreted from the pituitary upon stimulation of gonadotropin releasing hormone (GnRH). LH subsequently binds to the G-protein coupled surface receptor, LHCGR and activates the cAMP/PKA, MAPK and PI3K pathways (56). As well as being found on the cell surface of the Leydig cell of the testis and theca and granulosa cells of the ovary, cells in the adrenal cortex also express LHCGR and are able to respond to surges or losses of LH signalling (70, 71). This was demonstrated by the development of adrenocortical neoplasms following

gonadectomy (GDX) and has been demonstrated in a number a species including goat, ferret and certain strains of laboratory mouse (55, 56, 70). Experiments examining these neoplasms in the mouse and ferret have demonstrated that LH signalling plays an important role in adrenal remodelling. Additionally, the formation of ectopic gonadal like tissue in the adrenal cortex following GDX is viewed as an extreme example of adrenocortical remodelling (25).

1.2.5 IGF signalling

Insulin-like growth factor (IGF) has also been implicated in adrenocortical zonation and renewal and consists of two ligands, IGF1 and IGF2. These ligands are able to bind to a receptor tyrosine kinase known as IGF1R to regulate mechanisms such as mitosis and cell survival through the MAPK and PI3K pathways (50). *IGF1* and *IGF2* have been shown to be comparably expressed in the adult adrenal. However, IGFR1 has been shown to be localised in the subcapsular region of the adrenal cortex, and its activity is modulated by IGF binding proteins (50). Mouse models investigating the impacts of *Igfr1* ablation in the adrenal noted agenesis and male to female sex reversal. Interrogation of the adrenogonadal primordium of these knockout mice revealed significantly fewer SF1+ cells compared to wildtype mice and was determined as essential for adrenocortical cell specification (72). Furthermore, IGF signalling has also been demonstrated in enhancing steroidogenesis in the adrenal cortex (73).

1.2.6 TGFβ signalling

Experiments examining the role of Transforming growth factor β (TGF-β) have highlighted that this signalling pathway is important in the differentiation of stem/progenitor cells in the adrenal cortex (74). Due to the wide array of ligands that make up the TGF-β superfamily, this permits regulation and interaction with a multitude of pathways. Two important members in relation to adrenocortical renewal are activin and inhibin. Numerous experiments dissecting the roles of activing in the adrenal cortex have noted that activin can inhibit adrenocortical cell growth (54),

induce and enhance the clearance of the X-zone from the adrenal cortex and impact steroidogenesis (52). Studies investigating adrenal cortex inhibin deficient mice revealed that upon gonadectomy, tissue characteristic of ovarian cells accumulates in the adrenal cortex. The loss of *Inha* also demonstrates constitutive TGF-β2 activation in adrenal progenitor cells, which result in expansion of cells that express Gata4 and other gonadal markers. These studies highlight the importance of activin and inhibin in the maintenance of an adrenal cell lineage (52, 53).

1.2.7 FGF signalling

The fibroblast growth factor (FGF) signalling pathway has been shown to be important in adrenal cortex maintenance and homeostasis. Both the adrenal capsule and the adrenal cortex express FGF-2 and FGF-9 which signal to FGFR-1 and FGFR-2 (45). Studies that ablate *Fgfr2* in steroidogenic cells demonstrate adrenal hypoplasia that occurs early in embryonic development. The adrenal hypoplasia evident in *Fgfr2* ablated mice results from reduced cortical proliferation during embryogenesis. This study highlighted the importance of FGF-2 during adrenal development (75).

1.3 Transcription factors involved in cortex maintenance and remodelling

1.3.1 SF1

Steroidogenic factor 1 (*Sf1* also known as *Nr5a1*) is a well described and essential regulator of the adrenal cortex. It is integral for regulating genes required for development and steroidogenic cell function (76). SF1 is described as an orphan receptor, however recent studies have shown that certain sphingolipids and phospholipids are able to bind to and regulate SF1. The importance of *Sf1* signalling in the adrenal was demonstrated via *Sf1* null mice, these animals displayed degeneration of the adrenogonadal primordium (AGP) due to apoptosis and the failure to develop an adrenal or gonad (77). Additional studies that examined disruption to upstream regulators of *Sf1* (*Wt1*, *Pbx* and *Cited*) discovered impaired adrenal development (25, 62, 78). Further studies that examined $Sf1^{\pm}$ mice highlighted reduced adrenal size, impaired glucocorticoid synthesis under stress and failure to respond to

unilateral adrenalectomy, which usually sees a compensatory increase in organ size (79, 80). Conversely, studies examining the impact of *Sf1* overexpression in human adrenocortical cells via activated doxycycline treatment revealed that this drives cells through proliferation and negate apoptosis (81). An increase in S*f1* expression has also been observed in the development of childhood adrenocortical tumours (82).

1.3.2 Dax1

Dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X (*Dax1* also known as *Nr0b1),* is an X-linked gene which encodes a repressor of steroidogenic gene expression. This was demonstrated through examination of Y-1 mouse adrenocortical cells transfected with DAX-1. Steroidogenesis was supressed in these cells following transfection (60). In the adrenal cortex the activity of SF1 is modulated by *Dax1* (59). Upon stimulation of the adrenal cortex by ACTH SF1+ progenitor cells downregulate *Dax1* to be able to differentiate. Mutations in *Dax1* in humans and mice have shown to result in excessive differentiation and eventual depletion of the stem progenitor compartment (61).

1.3.3 WT1

Experiments analysing the fate of adrenocortical stem cell populations identified Wilms tumour 1 positive cells (WT1) as a long-lived progenitor population residing in the adrenal capsule. These cells were identified by expression of *Wt1* and *Gata4*, which are also markers of the AGP (83). The WT1+ cells become activated following gonadectomy, this results in the differentiation of cortical cells into a gonadal cell lineage (25, 83). These studies demonstrate the maintenance of a cell population in the subcapsular region with AGP properties. Repression of *Wt1* in the mouse embryo is required for the appropriate expression of *Sf1* and differentiation of stem cells into an adrenocortical cell lineage (84).

1.3.4 GATA6

GATA binding protein-6 (*Gata6)* is another transcription factor expressed in the adrenal cortex of the foetal mouse (85) and becomes localised to the capsule and subcapsular regions of the postnatal adrenal cortex. *Gata6* ablation in SF1+ cells results in cortex shrinkage, cytomegaly, blunted corticoid production and the

expression of gonadal markers (86). It was therefore hypothesised that GATA6 is required to limit the differentiation of adrenal stem cells into gonadal cell types. Zonation is also impacted following *Gata6* ablation: virgin females lack an X-zone and castrated males do not form a secondary X-zone (86). The reason for this is not clear as postnatal X-zone cells do not express *Gata6*. It was hypothesised that it was either a result of non-cell autonomous phenomenon or that it occurs in foetal adrenal cells that co-express *Gata6* and *Sf1*-Cre.

1.4 The foetal Adrenal: Development and Formation

The adrenal cortex starts to develop from a condensation of the coelomic epithelium in the embryo, called the urogenital ridge which also gives rise to the gonads and kidney. The steps of adrenal development are quite different in mice and humans. Remarkably, in both species the process of adrenal remodelling continues after birth.

1.4.1 Adrenogonadal primordium (AGP)

Multiple mechanisms and molecules are integral for the specific and timely development of the adrenogonadal primordium and separation from the developmental lineage of the kidney. Two in particular, SF1 and DAX1 are essential for the maintenance of the adrenal cortex as described in figure 1.3, but also for the development of the adrenal cortex and gonads. An identical precursor population for the adrenal and gonads was identified through staining for SF1, which revealed a population of positive cells located in the developing urogenital ridge from the coelomic epithelium to the dorsal aorta at embryonic day 11.5 of rat development (87). This cell population then differentiates into the adrenal and the gonadal primordia. The process of this differentiation begins upon contact with primordial germ cells. Depending on the sex of the organism the gonadal primordium goes onto form either the testis or ovary, and the adrenal primordium differentiates into the adrenal cortex.

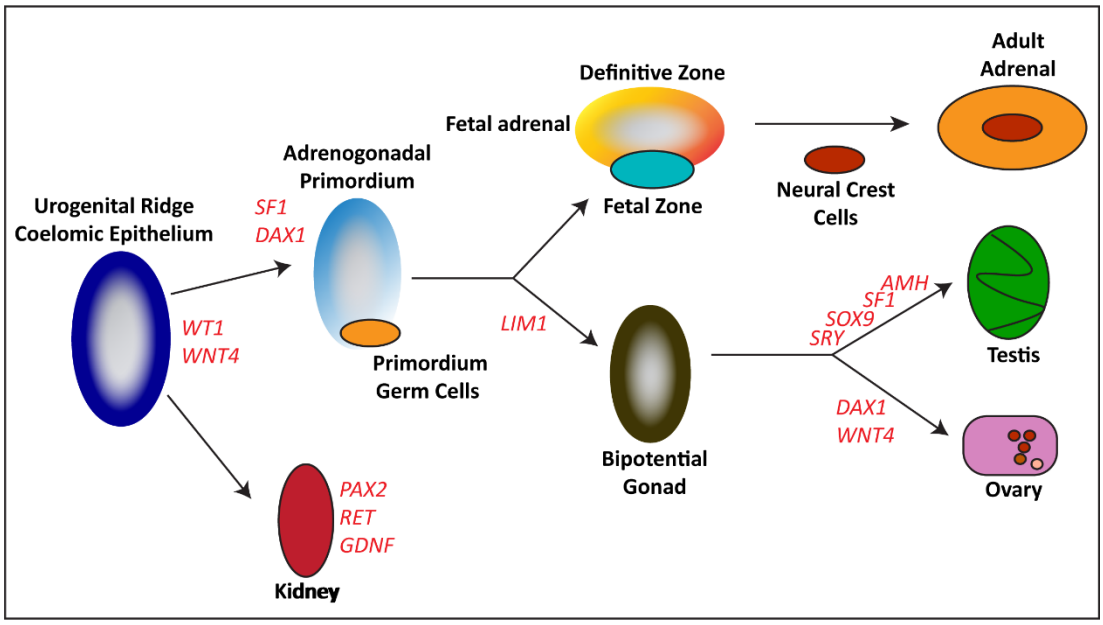


Figure 1-3. Development of the adrenogonadal primordium (AGP). *Diagram depicting the mechanisms driving the development of the adrenal cortex and gonad and the important genes required at key differentiation points are highlighted in red. Development of the urogenital ridge permits formation of the kidneys, gonads and adrenal cortex. Separation of the kidney and AGP is the first step. This is followed by contact of the AGP with primordial germ cells. This contact results in the separation of the developing adrenal and the bipotential gonad. The bipotential gonad will differentiate into a testis or ovary depending on sex. The adrenal cortex continues to develop with the integration of neural crest cells that form the adrenal medulla. These cells migrate to the centre of the adrenal gland, and the process of cortical zonation is initiated.*

1.4.2 Formation of the adrenal gland from the AGP

1.4.2.1 Human

Condensation of the coelomic epithelium is detectable between 3-4 weeks of gestation. During the 4th-6th week of development the migration and proliferation of the coelomic epithelial cells begins leading to the morphological differentiation of the two distinct foetal adrenal zones, termed the outer definitive zone (DZ) and the inner foetal zone

(FZ) at around 8 weeks of gestation. The foetal zone is steroidogenically active, producing high amounts of DHEA-S from large eosinophilic cells, which is converted into oestrogens by the placenta. The definitive zone is made up of a small layer of densely packed basophilic cells which are thought to be the reservoir of progenitor cells which will ultimately give rise to the defined cortex zones. By weeks 9-10 the adrenal primordium is now completely surrounded by the adrenal capsule. Approaching the end of gestation, a third layer of the adrenal cortex is now visible, termed the transitional zone. This zone is located between the and definitive zones (88). After birth the involution of the foetal zone occurs and the definitive zone then becomes the adult adrenal cortex.

1.4.2.2 Mouse

The adrenogonadal primordium can be observed by day 9 of embryonic development in the mouse (E9). As described, SF1 expression can identify these cells which go onto progress down either adrenal or gonadal lineages (84). This differentiation can be observed by E11.5-E12. By E12 the adrenal primordium becomes infiltrated by cells from the neural crest which will ultimately form the adrenal medulla. A distinct adrenal cortex and medulla can be observed from E16 in the mouse. From this stage, functional zonation of the definitive adrenal cortex can be observed which develops into the adult adrenal cortex. This differentiation of the cortex is defined by the expression of *Cyp11b2* integral for aldosterone synthesis by E19, which localise at the outer adrenal cortex and the presence of *Cyp11b1* expressing cells which localise in the inner part of the adrenal cortex. After birth, the commonly described rodent foetal zone, known as the X-zone becomes evident by day d10 and regresses differently in the male and female (Fig. 1-4). Males see X-zone regression by d35 and females see regression at first pregnancy (89). Although the timing and presence of the foetal zone/X-zone in the human and rodent differ, they are thought to be analogous and programmed by similar genes. .

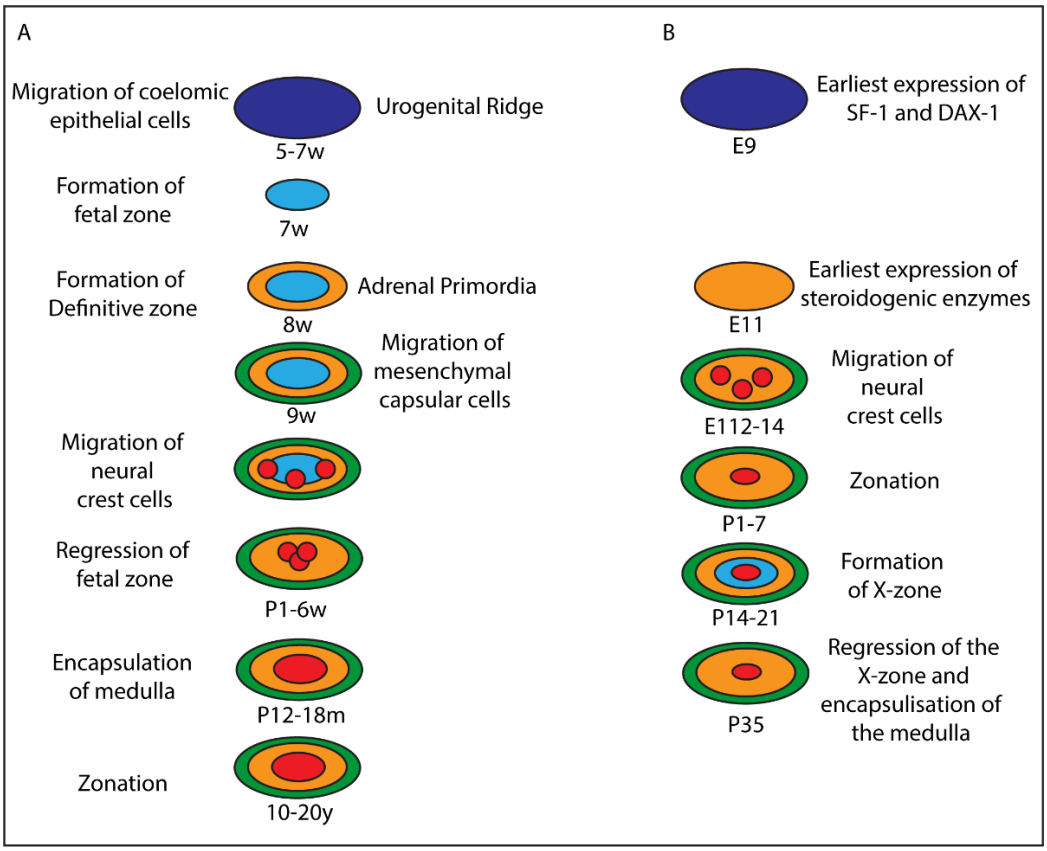


Figure 1-4. Development of the adrenal cortex from the AGP. *Diagram depicting the mechanisms driving the development of the adrenal cortex.* ***(A)*** *Human adrenocortical development starts from progression of the urogenital ridge to final zonation (dark blue zone). Two separate populations of cells migrate from the coelomic epithelium to form the foetal (light blue zone) and the definitive zones (orange zone) collectively termed the adrenal primordia. Mesenchymal cells then migrate to form the capsule (green zone) and finally neural crest cells migrate to the centre and form the medulla (red zone). The foetal zone regresses after birth.* ***(B)*** *Mouse adrenal development follows a similar process, however zonation is nearly complete by birth in rodents and the foetal zone (X-zone) appears postnatally and regresses in a sex dependent manner.*

1.4.4 Purpose of foetal cells and implications for adult cortex

In numerous mammalian species, the foetal adrenal cortex consists of the adult and foetal cortex zones whose origins and mechanisms of control are still not clearly understood. Studies examining the morphology of these zones have resulted in three current paradigms. The first of these hypothesises that the adult zone may be derived from the coelomic epithelia after cells that form the foetal cells have migrated (90), alternatively, the foetal and adult zones may derive simultaneously from discrete populations of the coelomic epithelial cells (91). The third model postulates that the zones both develop from a single progenitor population, which differs from the other two models which suggests the cell fates of these two populations are already decided upon migration from the urogenital ridge (92). These models were hypothesised in the late 20th century and with the complexity of adrenal development it was difficult to determine an exact mechanism. However, recent studies conducted examining lineage tracing during adrenal development have suggested that the third model is most convincing at this point in time through showing that the adult zone derives from an early stage of the adrenal primordium in which the foetal adrenal-specific enhancer (FAdE) is transiently activated.

Additional experiments conducted by Zubair *et al* demonstrated a foetal adrenal-specific enhancer of the *Ad4bp/SF1* gene which was shown to stain positive in precursor cells that contributed to both the adrenal and gonads (84). This study was also able to demonstrate the transient foetal X-zone. They observed the increase in cell number of the definitive cortex and the eventual regression of the X-zone and restriction to the cortex-medulla boundary, thus suggesting the mechanism for transition from the foetal to the adult cortex is governed by a similar mechanism across most mammalian species. This same group noted through the investigation of Ad4BP-LacZ-FAdE Tg mice, the presence of distinct population of cells between the foetal LacZ-positive cells and LacZ-negative adult adrenal cortex. This band of cells was lacZ negative, however, were morphologically similar to foetal cells. They noted that some of the cells making up this intermediate zone stained faintly for LacZ. The faint LacZ staining and morphology of the intermediate cells led to the hypothesis that this band of cells are transitioning from foetal to adult cells. Furthermore, they suggested

that there is simultaneously a change in enhancer in the Ad4BP/SF-1 locus from the FAdE to and adult adrenal enhancer which has still not been identified (93). Although further work would need to be carried out to confirm, Dax-1 has been identified as the gene potentially responsible for this switch, as models that ablate Dax-1 maintain foetal cells in the adult cortex (94).

1.4.3 Rodent X-zone

In mice, the foetal zone or X-zone can be observed histologically by d10 in postnatal development. Regression of this zone occurs in a sex dependent manner, with regression occurring during puberty in the male and during first pregnancy in females (34, 35, 95). If no pregnancy occurs this zone regresses with age in females. Several lines of inquiry have suggested that despite timing and developmental differences between the foetal cells in humans and rodents, that the two zones are analogous structures and are controlled via similar mechanisms. A number of similarities have been drawn between the two structures. They are located in the same position, adjacent to the adrenal medulla and both foetal cell populations contain ultrastructural features of steroid-producing cells. In addition, loss of DAX1 function in both humans and rodents results in a failure of regression of these foetal cells (94, 96, 97). Despite numerous studies investigating the X-zone, its origin and true function are still unknown, through continuing to identify new models to examine mechanisms controlling the formation and regression of the X-zone should help elucidate its function.

1.4.3.1 Androgens and the X-zone

Experiments conducted as early as 1937 by Deansley and Parkes demonstrated the impacts of androgens on the adrenal X-zone. They first demonstrated the regression of the mouse X-zone in untreated males during puberty and the redevelopment following castration, highlighting that the control of this regions was potentially andromimetic (98). Through a series of experiments, they also found that treatment with androgenic compounds shortly after birth and in response to castration prevented the development of an X-zone. Starkey and Schmidt later examined the effects of testosterone in both

males and females mice and demonstrated the failure to develop X-zones in both sexes following administration of testosterone in prepubertal mice (36). Experiments carried out by Jones demonstrated that the X-zone still regresses in hypophysectomised male mice given testosterone, highlighting that this regression is not under the control of the pituitary, but androgens work directly at the level of the adrenal to remove the X-zone (99). Despite this early identification of androgen action on the X-zone, the mechanism is still not understood and experiments dissecting this have not expanded on the issue much in recent years.

1.4.3.2 Recent advances in dissecting X-zone control and function

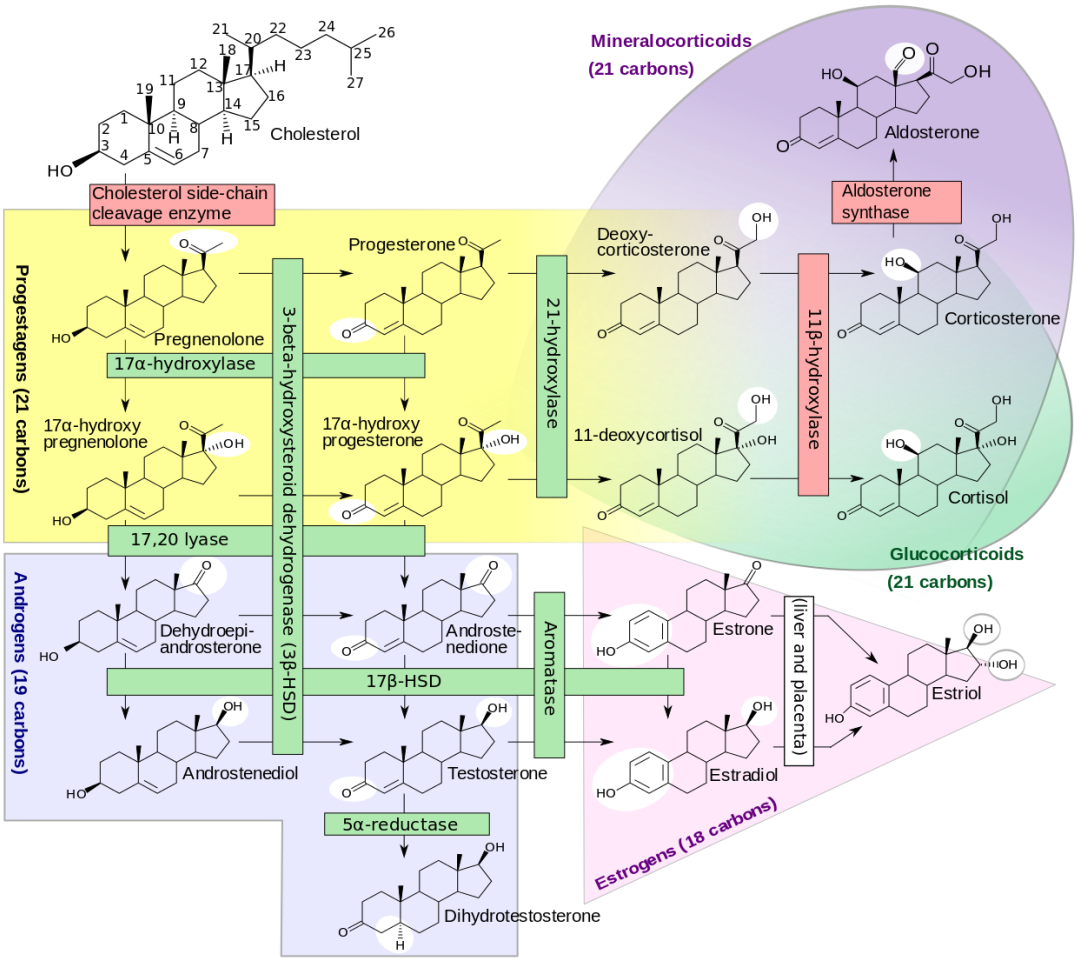
In addition to *Ad4bp/SF1*, *Dax1* and androgens being highlighted as regulators of the adrenal X-zone, additional knockout models have noted an impact on the rodent X-zone. Sahut-Barnola *et al* identified the presence of a mislocated X-zone in both males and females following the ablation of *Prkar1a* in the adrenal cortex, they noted that with age, the X-zone migrated from its normal location at the medulla boundary up through the cortex, highlighting that this pathway could be involved in controlling the foetal cell population (29). Another study performed quantitative trait locus mapping of the genes associated with the vacuolisation of the X-zone. They highlighted strain dependent differences in how the X-zone regresses and a selection of potential genes involved (35). Another study investigated the expression of thyroid hormone receptor beta (*Thrb*) in the adrenal cortex through expression of β-galactosidase. Through this model they identified a novel TRβ1-positive zone in the adrenal cortex that exhibits sexually dimorphic regression at the juxtamedullary region. They also demonstrated that treatment with T3 is able to induce a change in morphology of these cells suggesting that TRβ1 modulates inner cortical responses at sensitive postnatal time points. Furthermore, they showed that treatment with T3 maintained the presence of an X-zone after puberty if treated while the X-zone was still present, however if the X-zone was allowed to regress normally and treated with T3 the X-zone did not re-develop (40).

Chapter 1: Literature Review

1.5 Adrenal steroidogenesis

1.5.1 Adrenal steroid production

The production of adrenal steroids is a dynamic process that relies on de novo synthesis from cholesterol, under the control of ACTH and other regulators. The adrenal is responsible for the synthesis of mineralocorticoids, glucocorticoids and adrenal androgens and it does this in a zone specific manner. Each of the adrenals concentric zones expresses specific enzymes that enable production of particular steroids, discussed in the subsequent sections. Steroid biosynthesis begins via the mobilization of cholesterol from a pool in the outer mitochondrial membrane, which is continuously replenished from cytosolic storage droplets of cholesterol esters (3). The steroidogenic acute regulatory (StAR) protein facilitates cholesterol transfer from the outer mitochondrial membrane to the inner mitochondrial membrane, this enables side-chain cleavage enzyme CYP11A1 to catalyse the first and rate limiting step of steroidogenesis, the conversion of cholesterol to pregnenolone (100). Zone-specific steroid biosynthesis is described in the subsequent sections.



Key:
Mineralocorticoids – Zona Glomerulosa, Glucocorticoids – Zona Fasciculata, Androgens – Zona Reticularis, Estrogens – Peripheral tissue.

Figure 1-5. Steroid biosynthesis pathway. *Mineralocorticoid production (purple region) - Biosynthesis pathway of aldosterone production under the control of angiotensin II and high extracellular potassium in the ZG. Glucocorticoid production (green region) - Biosynthesis pathway of Glucocorticoid production upon stimulation of ACTH and negative feedback in the ZF. Primary glucocorticoid in humans is cortisol and corticosterone in rodents. Androgen production (blue region) - Biosynthesis pathway of androgen production upon stimulation of ACTH in the ZR. Conversion of androstenedione and testosterone to Estrogens (pink region) occurs in periphery tissues such as the liver and placenta. Adapted from (101)*

1.5.1.1 Mineralocorticoid production and targets

Mineralocorticoid production occurs in the zona glomerulosa which is characterised by three distinct features; it expresses angiotensin II receptors, along with the expression of P450c11AS, but does not express P450c17. For these reasons the glomerulosa is able to exclusively produce aldosterone under the regulation of the renin/angiotensin system (102). Presence of angiotensin II and high extracellular potassium are the main stimulators of aldosterone synthesis through increased intracellular calcium. Synthesis from pregnenolone requires the action of three enzymes. 3β-hydroxysteroid dehydrogenase type 2 (HSD3B2) in humans and type 1 in rodents (HSD3B1) performs the irreversible conversion of the hydroxyl group to a keto group on carbon 3 and simultaneous isomerization of the double bond from the Δ^5 to the Δ^4 position, converting pregnenolone to progesterone (103). Secondly, 2.21-hydroxylase (CYP21A2, P450c21) converts progesterone to 11-deoxycosticosterone and finally, aldosterone synthase (CYP11B2, P450c11AS) then catalyses the final reactions; 11β-hydroxylation, 18-hydroxylation, and 18-methyl oxidation to aldosterone. The 18-aldehyde group, from which the name "aldosterone" derives, forms an intramolecular cyclic hemiacetal using the 11β-hydroxyl group, with loss of water (104). Once released from the adrenal, aldosterone plays a pivotal role in electrolyte and fluid homeostasis and therefor is essential in the control of blood pressure. Aldosterone has been shown to target the epithelial of the distal colon and renal nephron. Upon stimulation, it results in sodium reabsorption and potassium secretion (105).

1.5.1.2 Glucocorticoid production and targets

The zona fasciculata does not express angiotensin II receptors or P450c11AS so cannot produce aldosterone, instead it expresses the ACTH receptor MC2R and P450c11β. In the ZF, CYP17A1 is also able to catalyse the 17α-hydroxylation of pregnenolone and progesterone. From this step CYP17A1 is then able to cleave the C17-C20 bond of the 17-hydroxypregnenolone and 17-hydroxyprogesterone (17OHP). This step does not

occur in the ZG due to no CYP17A1 expression. This cleavage results in 19-carbon steroids and occurs at a single active site but with different regulation. HSD3B2 and CYP21A2 perform much the same way as they do during the synthesis of aldosterone, 17-hydroxysteroids are converted to 11-deoxycortisol. Steroid 21 hydroxylase (CYP21) is an enzyme that is unique to the adrenal cortex and permits the biosynthesis of mineralocorticoids and glucocorticoids as well as androgens (106). In the final step 11β-hydroxylase (CYP11B1, P450c11β), an enzyme which has been shown to be closely related to CYP11B2, completes cortisol synthesis. P450c11β is not able to convert 18-hydroxycorticosterone to aldosterone, additionally, it has minimal capacity to convert corticosterone to 18-hydroxycorticosterone (102). In mice, and many small rodents, the zona fasciculata lacks CYP17A1 expression and as a result corticosterone is the dominant glucocorticoid. Humans only produce a small amount of corticosterone as cortisol is the dominant glucocorticoid (107, 108). Once released into the circulation, glucocorticoids regulate a number of biological systems most notably the hypothalamic-pituitary-adrenal (HPA) axis and are essential in regulating the body's response to acute and chronic stress (109). Discussed in more detail in section 1.6. Glucocorticoids exert their effects through their receptor glucocorticoid receptor, discussed in detail in section 1.6.2.

1.5.1.3 Adrenal androgen production and targets

MC2R is also expressed in the zona reticularis, however with minimal P450c21 or P450c11β only a small amount of cortisol is produced. This zone however, does express large amounts of P450c17 and cytochrome b_5 (107). The expression of cytochrome b_5 maximises 17,20-lyase activity and production of C_{19} steroids. Dehydroepiandrosterone (DHEA) and its sulfate (DHEAS) are the two most abundant adrenal steroids that are produced. The production of DHEA from pregnenolone and production of androstenedione from progesterone is catalysed by a single enzyme, CYP17A1. Despite the presence of CYP17A1 in both the zona fasciculata and zona reticularis, through the action of cofactor cytochrome b_5 (CYB5A) in the ZR the 17,20-lyase reaction is enhanced approximately 10 fold (110). The lack of CYP17A1 in the

mouse adrenal not only results in the inability to produce cortisol, it also means that the mouse adrenal does not produce androgens (111). DHEA is conjugated to DHEAS through sulfotransferase SULT2A1 which is essential in regulating the synthesis of adrenal androgens. In addition to the production of DHEAS, the adrenal is able to synthesise small amounts of testosterone through via the conversion of androstenedione by 17β-hydroxysteroid dehydrogenase type 5 (17βHSD5, AKR1C3) (112). Stimulation of androgen production from the zona reticularis is primarily by ACTH. The zona reticularis, as previously described, bears similarity to the foetal zone during foetal development. The foetal adrenal is responsible for the production of the C_{19} substrate for oestrogen synthesis during pregnancy but involutes quickly after birth. The zona reticularis forms only a small layer in the adrenal during infancy, however expands throughout childhood leading to increased levels of circulating DHEAS and the process of adrenarche. Adrenarche is characterised by the development of axillary hair and pubic hair in response to the peripheral conversion of adrenal androgens to more potent androgens. Production of DHEAS peaks around 25 years of age and gradually declines with age (113). In adulthood, circulating androgens produced by the adrenal only account for less than 5% of the total circulating androgens in men compared to the production of androgens from the testis. However adrenal androgens in women account for around 25% of total circulating androgens (114). Inappropriate action or production of adrenal androgens has been recognised in a number of clinical conditions such as premature adrenarche, castration-resistant prostate cancer, congenital adrenal hyperplasia and polycystic ovarian syndrome (115). Androgens exert their effects through their cognate receptor, androgen receptor (AR), discussed in detail in section 1.6.1.

1.5.1.3.1 Gonadal androgen production

Androgen production not only occurs in the adrenal gland, it is also produced in the Leydig cells of the testis and thecal cells in the ovary. Androgen biosynthesis occurs as described in the previous section and in figure 1-5. Gonadal androgen secretion however is under the control of the hypothalamic-pituitary-gonadal axis.

Chapter 1: Literature Review

Gonadotropin releasing hormone (GnRH) stimulates luteinizing hormone (LH) release from the pituitary which targets the gonad to produce androgens and work in a negative feedback loop at the hypothalamus and pituitary.

1.6 Steroid Receptors

Steroid hormone receptors belong to the nuclear receptor family. These receptors are ligand-regulated transcription factors activated through the binding of their specific hormones. The steroid ligands for these receptors are lipid soluble and so are able to cross the plasma membrane to interact with the nuclear receptors directly and do not need to be transported through cell surface receptors (116). Unbound steroid receptors are sequestered in the cell cytoplasm. When their ligand is bound, nuclear receptors undergo a conformational change, lose bound heat shock proteins, dimerise and translocate from the cytoplasm to the nucleus to directly control the transcription of genes and regulate many cellular processes such as reproduction, cell proliferation, metabolism and development. The primary role of nuclear receptors are as transcription factors, however, they have also been shown to be able to regulate cellular processes in the cytoplasm. For the majority of nuclear receptor specific ligands have been identified, however, there are a number of receptors that do not have an identified ligand, these are termed 'orphan receptors'. Whether these receptors have specific ligands is unclear, as some nuclear receptors are able to regulate transcription in the absence of a ligand (117). AR and GR structure and function are described in detail in the following sections.

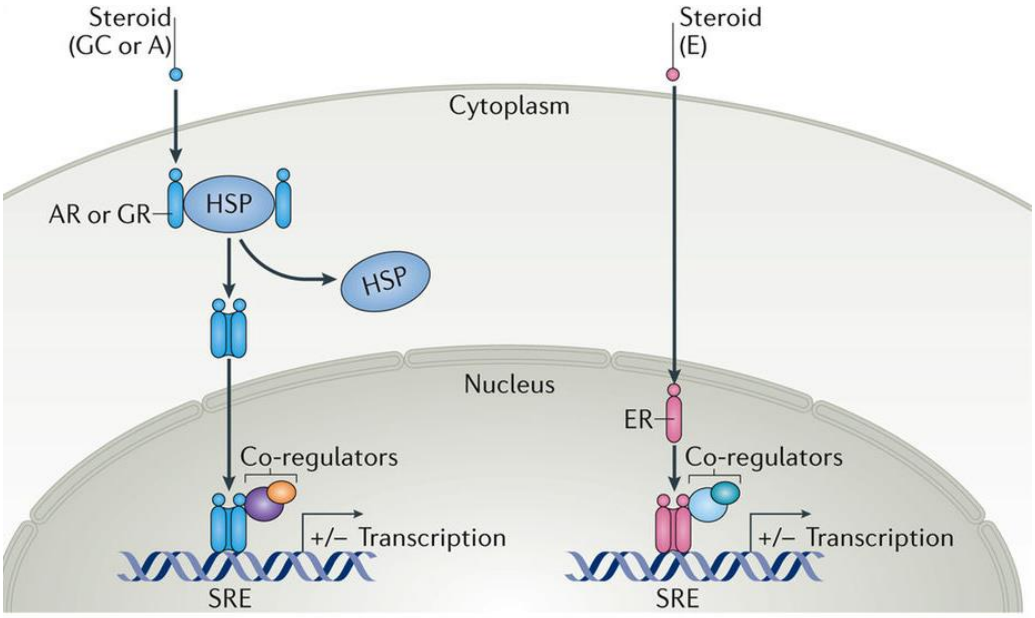


Figure 1-6. Diagram showing basic nuclear receptor signalling (118). *Steroid receptors, such as glucocorticoid (GC) and androgen (A) receptors, are primarily in the cytoplasm as monomers bound to heat shock proteins. Others, such as the oestrogen (E) receptor (ER), are located as monomers primarily in the nucleus. Steroid binding to cytoplasmic receptors triggers release from the HSPs, receptor dimerization, alterations in receptor conformation and nuclear localization. In the case of E, the sex steroid binds to nuclear receptors to promote dimerization and changes in receptor conformation.*

1.6.1. Androgen Receptor

1.6.1.1 Androgen receptor structure

The Androgen receptor gene, also known as NR3C4 (nuclear receptor subfamily 3, group C, gene 4) is a nuclear transcription factor and is a member of the steroid hormone receptor superfamily of genes. AR was first characterised by Migeon *et al* in 1981 and is located on the X-chromosome at q11-12 and consist of 8 exons (119). AR, like all steroid nuclear receptors, comprises of four different domains that are structurally and functionally distinct. These are the DNA-binding domain (DBD), which is highly conserved, the ligand-binding domain (LBD), which is moderately

conserved, and the N-terminal domain (NTD), which is poorly conserved between other members of the steroid family. Another region called the hinge region, is a short amino acid sequence which separates the LBD and DBD. This region contains a bipartite ligand dependent nuclear localisation signal (NLS) which is used for AR nuclear transport (120).

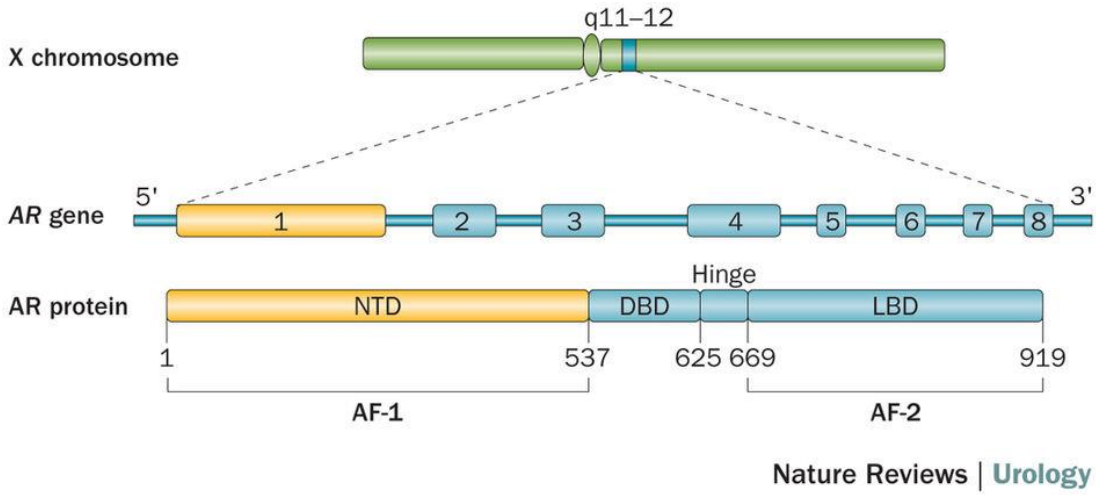


Figure 1-7. Diagram detailing AR gene and protein (121). *AR is located on the X chromosome and consists of eight exons. These code for the NTD, DBD, hinge region and LBD. The AR contains two transactivation domains, termed AF1 (activation function 1) located in the NTD and AF2 (activation function 2) in the LBD. AF2 exhibits weak transcriptional activity, whereas AF1 is a strong regulator of transcription.*

The NTD is the region that acts as a binding site for other proteins in the transcriptional regulation complex. It is coded by exon one and is thought to be able to work independently of androgen stimulation as it was shown to regulate transcription in LBD-mutant mice. (122). Within this domain are two transcription activation regions, termed TAU-1 and TAU-5 (123). Experiments examining these two transcriptional activation units has noted TAU-5 as being responsible for the majority of the constitutive transcriptional activity within the NTD.

The DBD is coded for by exon 2 and 3 and is composed of two zinc finger motifs. One of the zinc fingers contains a P-box motif that co-ordinates recognition of DNA sequences and the second contains a conserved D-box motif which helps stabilize DNA-receptor interactions through interacting with the phosphate backbone and varies between different transcription factors to ensure specific binding to particular steroid receptor response elements (124, 125). The hinge region separating the LBD and DBD is approximately 50 amino acids and contains the NLS essential for AR nuclear transport. This transportation of AR into the nucleus is facilitated by filamin-A, a cytoskeleton protein (126). Interrogation of AR transportation into the nucleus of filamin-A negative cell lines revealed the failure of AR nuclear transport, even following prolonged exposure to androgens (127).

The LBD is responsible for facilitating the binding of ligands testosterone and DHT to AR, this is the primary mechanism for androgen signalling. In the LBD, AF2 interacts with LxxLL-containing co-regulators, these co-regulators have been identified as the steroid receptor coactivator [SRC] /p160 family(128). LBD contains another binding region specifically for heat shock protein 90 (HSP90), which is common to all steroid hormone receptors. The primary role of HSP90, as well as other heat chock proteins is to prevent interaction of AR with its DNA response elements27, line in the absence of its ligand, in addition to maintaining AR structure (129).

1.6.1.2 Classical mechanisms of AR signalling

Both testosterone and the more potent DHT are able to bind to AR and initiate the regulation of transcription. DHT binds with higher affinity to AR compared to testosterone and can activate target genes in lower concentration than testosterone. In the absence of a ligand, AR is primarily located in the cell cytoplasm where, as previously mentioned, it is bound with heat shock proteins. Upon ligand binding, dissociation of heat shock proteins occurs and induction of a conformation change in AR takes place where by helices 3, 4 and 12 the LBD go onto form the AF-2 binding surface and the unmasking of the nuclear localisation signal. The positioning of helix 12 across the ligand-binding pocket reduced ligand dissociation (130). Subsequent

hyper-phosphorylation of AR occurs and induces the recruitment of co-activators and the activation of transcription. This complex then translocates to the nucleus (131). Dimerization of the ligand-receptor complex is required to permit binding to androgens response elements (ARE), these are located in the regions of androgen responsive genes. The binding of AR to their specific AREs permits the recruitment of histone acetyltransferase (HAT) enzymes, which are a range of co-regulators and the necessary machinery for transcription. There are two types of AREs, classical which are characterised as being 15bp in length and contain two 6 imperfect bp repeats. This is very similar to the response elements for glucocorticoids which permits activation of these AREs by GR to enable gene transcription. There are also selective AREs which are only androgen responsive (132, 133). For AREs to be able to modify transcription, they first need to form AR/ligand homodimers, this results in the recruitment additional proteins to help form a transcriptional activation or repression complex at discrete sites on the chromatin. Cofactors that are recruited to the complexes are thought to be cell response specific, this is due to the wide array of cell responses AR can mediate and control and stops off target or inappropriate transcription (134).

1.6.1.3 Non-classical mechanisms of AR signalling

As well as genomic roles of AR signalling, there has been mounting evidence for the non-genomic actions of AR signalling (135). AR non-genomic signalling occurs very rapidly and has been shown to occur from seconds to minutes. This extremely quick response time shows that it does not require transcription and translation from binding to response elements. There is a disparity between the wide array of androgen targeting effects and the lack of identification of AREs, and it is thought that the non-genomic actions of AR signalling can account for this discrepancy. Non-genomic AR action occurs in the plasma membrane or the cytoplasm, this activation results in the release of intracellular release of calcium and the activation of protein kinases including MAPK (ERK), protein kinase A (PKA), Akt and protein kinase C (PKC) (135-137). There has been some data to suggest that the activation of these pathways is able to feedback upon AR. Furthermore, Phosphorylation of ligand and non-ligand bound AR

will prevent the degradation of AR and can enhance translocation and transcriptional activity (138). The majority of non-genomic AR signalling will initiate signalling cascades that can regulate other nuclear receptors. Although there is not extensive literature demonstrating non-genomic AR signalling, it has been noted that the most common characteristic in non-genomic effects of exposure to androgens is rapid changes to calcium (Ca^{2+}) (135, 139). Modulation of calcium is a rapid process that occurs within seconds. For these reasons, it has been postulated that androgens must bind to a receptor on the surface of the cell. It has been noted that not all cell types that demonstrate a rapid androgen response, express classic nuclear androgen receptor or are able to be blocked by AR antagonists. It is currently unknown if the receptor located at the cell surface is the classical intracellular AR coupled to other signal transduction machinery or if it is a unique protein capable of binding androgens and initiating signalling cascades (140, 141). There has also been experiments conducted that demonstrate AR ligand independent signalling, this has been quite commonly observed in the development of castration resistant prostate cancer and was characterised by constitutively-active splice variant of AR that lack the LBD (142-144). Additionally, G protein-coupled receptor GPR6A has recently been reported to regulate extranuclear androgen-induced kinase signalling in prostate cancer cells (145). AR has also been shown to be activated by growth factors and proinflamatory cytokines in the absence or low concentrations of androgens (146, 147).

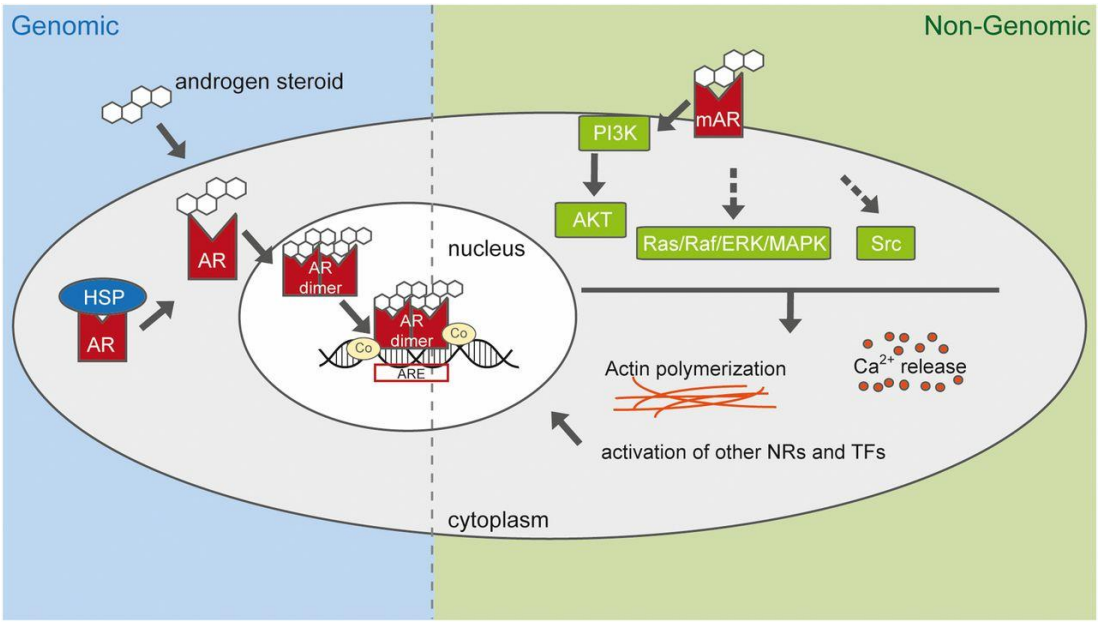


Figure 1-8. Genomic and non-genomic actions of androgen signalling (148) *Through the genomic pathway, androgens can move into the cytoplasm and bind to AR. This induces a conformational change and releases AR from heat shock proteins. Translocation to the nucleus occurs next, where AR dimerizes and interacts with cofactors and subsequently binds to androgen response elements enabling regulation of target genes. Non-genomic actions are thought to occur through a membrane-bound AR or other receptors, which then transduce the signal into the cytoplasm, interacting directly with other membrane bound factors such as PI3K or activating other pathways. This leads to changes in second messengers, cytoplasmic calcium concentration, actin skeleton changes, and eventually to the activation of other nuclear receptors and transcription factors.*

1.6.1.3 Androgen manipulation

Due to the wide array of mechanisms androgens and AR control, there have been numerous models in which to investigate their mechanism of action. This comes in the form of drugs that are able to target androgen receptor, GDX, naturally occurring mutations in AR and genetic manipulation through Cre/*loxP* technology (149). Drug treatments to block AR function has been achieved through the use of flutamide, which

is a non-steroidal antiandrogen (150). Used for a number of purposes, flutamide treatment has been shown to be effective in reducing adrenal androgen excess in congenital adrenal hyperplasia (CAH) (151) and in prostate cancer (152). An interesting study that investigated the impact of flutamide on the adrenal cortex following haemorrhaging in rats described restored cell structure and reduced tissue adenoma in the adrenal, this is thought to occur due to overcoming depressed adrenal function induced by severe trauma. However, controls in this study did not include a non-haemorrhaged rat treated with flutamide therefore, the impact on the adrenal cortex under normal conditions following flutamide treatment has not been described in this study (153).

Castration has also been widely used to investigate the impact of androgen loss on various tissues. Following castration, the adrenal has been shown to re-develop the X-zone, or, if this castration occurs before puberty, maintain the X-zone into adulthood (23). In addition to X-Zone re-development, the adrenal has also been shown to develop adrenocortical tumours (55, 56), the mechanisms underpinning this still aren't clear however, studies have shown that the loss of androgens results in a chronic elevation in LH and is thought to drive neoplastic formation in the adrenal cortex and expression of LHR (154). Additionally, treatment with testosterone in GDX mice show removal of the X-zone (36). The impacts of GDX on the adrenal cortex in various ablation models and the X-zone has also been discussed in section 1.4.3.1.

Models investigating natural mutations, such as the testicular feminized (*Tfm*) mouse which results in androgen insensitivity and feminised males. Interrogation of the adrenals in these mice noted the presence of an X-zone that had also been shown to be regulated by androgens in other models, demonstrated the normal involution of the X-zone in *Tfm* mice. This study suggested that the X-zone regression may not be entirely dependent upon androgen regulation (155).

A severe genetic mutation in humans known as Complete Androgen Insensitivity Syndrome (CAIS), arises due to an androgen receptor defect and prevents cells being able to respond to androgens (156). Although the impact of this disorder has been extensively researched, its impact on the adrenal cortex has not been described. Other

models that have described low circulating androgens is the hypogonadal (hpg) mouse (157), which are sexually immature and have arrested germ cell development, but again, these studies have not described or investigated the impact on the adrenal cortex.

In contrast to models that create low circulating androgens, treatment with human chorionic gonadotropin (hCG) has been shown to increase serum testosterone (158). Although this could be useful to investigate high levels of circulating testosterone in rodents and their impact on the adrenal, caution would need to be taken as the adrenal cortex has been shown to respond directly to hCG treatment (159). Ascertaining the adrenal phenotype as a result of high testosterone or direct hCG stimulation would be difficult.

1.6.1.3.1 Genetic rodent models of AR adrenal manipulation

A number of models have been generated to examine the impact of AR ablation in the rodent. These models range from global ablation of AR and cell specific models. Global Cre/loxP models were generated through the use of various constitutive promoters driving Cre recombinase, these included the use of an ACTB, CMV or PGK promoter to achieve global AR ablation. The male mice from these studies mimicked the phenotype seen in the testicular feminisation mouse displaying disruption of male specific genital structures, cryptorchidism, infertility due to spermatogenic block and a feminised external appearance. The females from these studies were not as severely affected but did have prolonged oestrus cycles and sub-fertility (160, 161). Very few of these studies have investigated the impact of AR ablation in the adrenal cortex of these mice, however one study investigating the impact of loss of AR in the pituitary of global knockout mice noted a disrupted adrenal with impaired cell death and presence of an X-zone. However, this phenotype was thought to be a result of a disruption in normal pituitary signalling (162). Cell-specific androgen receptor knockout mouse models have been generated that target many different cell types in the body including the testicular Leydig cells (163), Sertoli cells (164), and peritubular myoid cells (165), but thus far none have been generated to specifically investigate AR signalling in the adrenal. This list is not exhaustive however, highlights a lack of

knowledge of the role of autocrine AR adrenal signalling and its regulation of the adrenal cortex.

1.6.2 Glucocorticoid Receptor

1.6.2.1 Glucocorticoid receptor structure

As previously mentioned, glucocorticoids mediate their effects through their receptor glucocorticoid receptor (GR NR3C1). Like AR, GR is also a member of the nuclear hormone receptor family of ligand-dependent transcription factors (116). Human GR was first characterised through expression cloning in 1985 (166), is located on exon 5 and consists of 9 exons. GR has been said to repress the transcription of genes that account for 10-20% of the human genome (167-169) and is expressed in nearly every cell type in the body (170). The impacts of glucocorticoid signalling are extremely diverse and exhibit a varying response in tissues and across individuals. Recent studies have highlighted expanding roles for the various isoforms of GR which is thought in part, to permit the varying response to glucocorticoids, in addition to ligand bioavailability, and cofactor expression. Despite being derived from a single gene, GR has multiple proteins due to splicing and alternative post-translational regulation to further refine signalling properties (171, 172). Furthermore, the intracellular response to glucocorticoids is mediated by the complement of GR isoforms present.

Exon 9 is subject to alternative splicing (Fig. 1-9), which generates multiple GR splice variants, two of which, GRα and β, have been the primary focus of most studies due to their relative abundance. However GRβ is unable to bind glucocorticoids owing to an altered ligand-binding domain and has a more tissue-selective distribution. However, translation reinitiation that occurs at seven internal AUG codons in GRα mRNA, generates eight known receptor peptides with varying lengths of the N terminus. These receptor isoforms are designated GRα-A, -B, -C1, -C2, -C3, -D1, -D2 and -D3 (173). It is thought that the range of isoforms allows for the tissue specific and wide varying roles GR plays within the body.

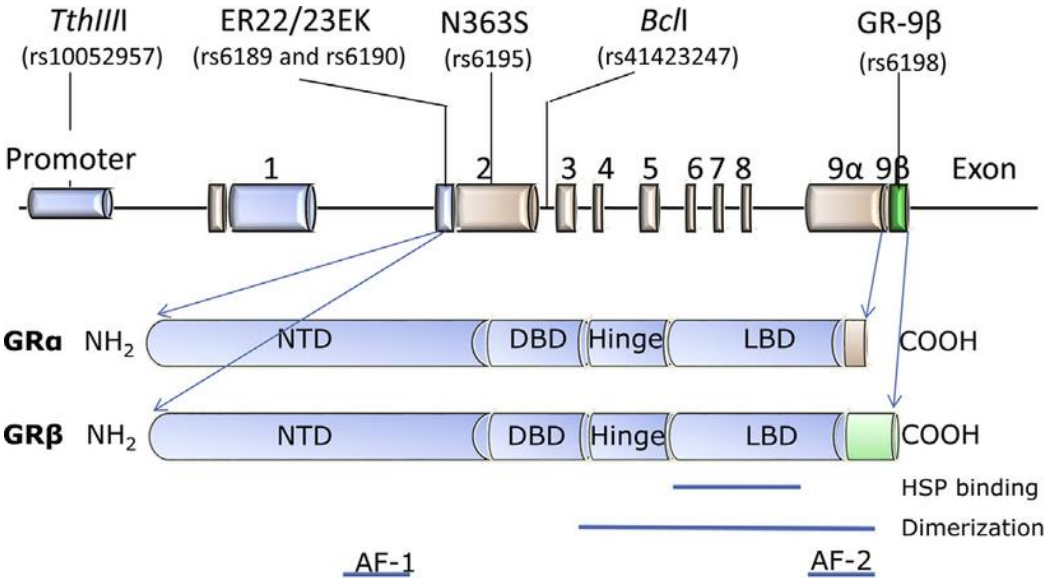


Figure 1-9. Diagram detailing Glucocorticoid Receptor gene and functional isoforms (174). *GR is located on chromosome 5q31-32 and is comprised of over 140 kb of nucleotides, less than 2% (~2.5 kb) of which make up the 9 exons. Alternative splicing of exon 9 results in the isoforms GRα and GRβ.The exons code for the NTD, DBD, hinge region and LBD. The GR contains two transactivation domains, termed AF1 (activation function 1) located in the NTD and AF2 (activation function 2) in the LBD. AF2 exhibits weak transcriptional activity, whereas AF1 is a strong regulator of transcription. GR undergoes multiple posttranslational modifications including phosphorylation, SUMOylation, ubiquitination and acetylation.*

Like AR, glucocorticoid receptor consists of a NTD, DBD, and an LBD separated by the hinge region. The DBD contains the zinc finger motifs essential for the recognition and binding to target DNA sequences named glucocorticoid response elements (GREs). The NTD contains AF-1 which is responsible for interacting with co-regulators and basal transcriptional machinery and is a target for post-translational modification. The LBD is the region that consists of 12 α-helices and four β-sheets (175). These structures form the hydrophobic pocket for binding to glucocorticoids

and contain AF-2 that interacts with co-regulators in a ligand dependent fashion. Nuclear localisation signals NL1 and NL2 can be found in the DBD hinge junction and LBD (176).

1.6.2.2 Classical GR signalling

When there is no ligand present, GR resides as part of a large complex of heat shock proteins (HSP90, HSP70, and P23) and immunophilins of the FK506 family (FKBP51 and FKBP52) in the cytoplasm (177, 178). This complex aids in maintaining receptor conformation and ensures inactivation of the receptor, to ensure no signalling occurs without the presence of a high affinity ligand. Circulating corticosterone is found bound primarily to corticosteroid-binding globulin (CBG), this is essential for the distribution and cell entry of glucocorticoids. Cortisol that is CMG-free has the ability to passively diffuse across the call membrane (Fig. 1-10), however, its ability to act upon cellular processes is mediated by two enzymes, 11β-Hydroxysteroid dehydrogenase type 2 (11β-HSD2), this enzyme oxidizes cortisol into the inactive metabolite cortisone and the second working in opposition, 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) converts cortisone to cortisol (179). Upon ligand binding to GR, structural changes result in NLS exposure and GR is rapidly translocated to the nucleus. Once inside the nucleus, GR is then able to bind to its GREs and regulate target genes. GR binds to GREs as a homodimer, studies have shown that GREs can not only positively regulate transcription, but can also negatively regulate it, these are known as positive and negative GREs respectively (180, 181). Binding initiates the control of transcription by facilitating the formation of the transcription initiation complex, which includes RNA polymerase II and its ancillary components via its AF-1 and AF-2 domains. Transcriptional initiation complexes work with numerous co-activators which include the p300/CBP-associated factor (p/CAF), which interacts with p300/CBP, but is also a broad transcription coactivator; and also the p160 family of coactivators, which interact with the steroid hormone receptors (182). The p160 coactivators interact with AF-2 through multiple amphipathic LxxLL signature motifs located in their nuclear receptor-binding (NRB) domain (183). These coactivator additionally facilitate HAT activity, promoting chromatin decondensation

and allows transcription (184). GR can also impact signal transduction cascades through protein-protein interactions with transcription factors and is thought to be able to stimulate or inhibit the rate of transcription of those target genes. The protein-protein interactions of GR with other transcription factors may take place on the promoters that do not contain GREs (described as the tethering mechanism), in addition to the promoters that have both GREs and responsive elements of transcription factors that interact with GR, described as composite promoters (185).

1.6.2.3. Non-classical GR signalling

GR is also able to mediate cellular responses via a quick non-genomic signalling mechanism, which can occur in minutes to seconds and does not involve transcription. This signalling mechanism has been shown to act on kinases PI3K, AKT, and MAPKs (186). This has been demonstrated in glucocorticoid-dependent release of accessory proteins associated with unbound GR in the cytoplasm, such as tyrosine kinase c-Src. Release of c-Src initiates multiple kinase cascades that lead to the phosphorylation of annexin 1, inhibition of cytosolic phospholipase A2 activity, and impaired release of arachidonic acid (187). Additional experiments have identified co-localisation of GR at the plasma membrane caveolae via an interaction with caveolin1 (188). Non-genomic actions of GR in the membrane has been shown to regulate gap junction intercellular communication and neural progenitor cell proliferation by non-genomic mechanism (189). The mechanisms by which GR controls transcription and cellular signalling is extremely diverse and understanding the different mechanisms by which GR mediates target genes is essential for understanding glucocorticoid meditated diseases and potential therapeutics.

1.6.2.4 Mineralocorticoid & GR Signalling

Glucocorticoids are not only able to mediate transcriptional regulation through GR but also mineralocorticoid receptor (MR). It is thought that the genes for MR and GR originate from a common ancestor prior to the evolution of aldosterone (190). Corticosteroids are essential in regulating various homeostasis mechanisms, acting through GRs and MRs, so it is therefore essential that there is a balanced activation of

these receptors to maintain health. Both mineralocorticoids and glucocorticoids bind to MR with equal affinity (172). Circulating cortisol is 100-1000 fold higher than those of aldosterone, so mechanisms are in place to prevent body wide MR being saturated by cortisol. This is achieved via the enzyme 11 beta-hydroxysteroid dehydrogenase type 2 (11bHSD2) (191). It works by converting cortisol and corticosterone into cortisone by a NAD (Nicotinamide Adenine Dinucleotide)-dependent manner. For many years it was thought that MR was purely localised to polarized tight epithelia, where it was co-localised to with 11bHSD2 to control aldosterone-dependent transepithelialsodium transport. However, studies in recent years have highlighted MR expression in non-epithelial tissue such as the hippocampus and the hypothalamus as well as many others. It has also been highlighted that MR as well as GR function is important in psychiatric conditions such as major depression (190).

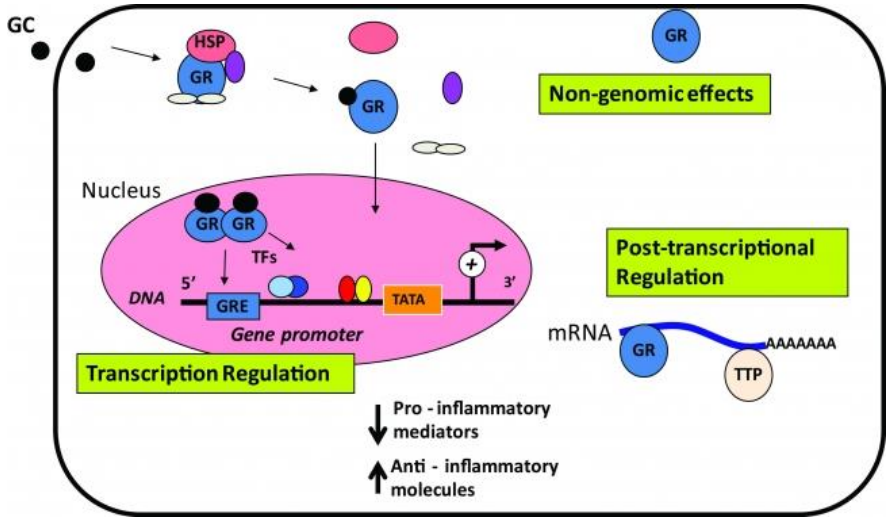


Figure 1-10. Genomic and non-genomic actions of glucocorticoid receptor signalling (192). *Glucocorticoids (GC) diffuse across the cell membrane where they bind to the glucocorticoid receptor (GR). Binding causes conformational changes which permits the release of the receptor from an inactive complex bound by heat shock proteins and translocates to the nucleus. The receptor dimerizes and can bind to DNA via GREs, or can interact with transcription factors to regulate their function. The receptor can also act post-transcriptionally to regulate RNA stability and via non-genomic mechanisms*

1.6.2.5 Rodent models of GR manipulation

Due to the presence of GR in nearly every cell type in the body an extensive list of GR ablation models has been curated. One of the first models of complete GR knockout described ablation of GR through the insertion of a neomycin cassette to exon 2 by homologous recombination in ES cells, thereby interrupting GR gene transcription in all cells of the body (170). Global GR ablation was achieved through the PGK promoter, results from this study highlighted 100% perinatal mortality rate due to retarded lung development and respiratory failure. The livers of these mice were not appropriately able to deal with key gluconeogenic enzymes and feedback of the HPA axis was severely impaired with significant increases in adrenocorticotropic hormone and corticosterone, which coincided with a hypertrophic adrenal gland. Another method to target GR was developed by Ridder *et al*, which generated a mutant mouse containing loxP sites surrounding Exon 3, a region involved in DNA binding. They reported similar findings to Cole *et al(193)*. A final allele developed by Brewer *et al* also utilised a floxed GR transgene with *loxP* sites that flanked exons 1C and exon 2 (194). The phenotype observed in these mice were similar to what was observed in Cole *et al*'s global knockout model. Due to the severity of the phenotype observed in GR global knockout mice, more refined methods were developed to investigate cell specific ablation of GR. Tronche *et al* generated a conditional GR knockout model in which exon 3 was floxed and excision was permitted through the use of a Nestin Cre to target cells in the nervous system (195). Additional tissue specific targeting of GR has been achieved through GR DNA binding mutants (196), pan-neural GR conditional knockouts (195), and forebrain GR (197) conditional knockouts, all showing severe defects and highlighting the importance of GR signalling in the respective tissues. In addition to loss of function analysis, mouse models were also used to examine overexpression of GR. This model was generated by Reichardt *et al* and involved the insertion of an extra copy of the full length GR gene through the use of yeast artificial chromosomes and increased protein expression by about 50%. This models permitted the investigation of GR overexpression and its impact on stress related behaviour (198). Additional tissue-specific over-expression models were generated to permit investigation of the forebrain and the overexpression in brain

structures such as the cortex, hippocampus, CeA, BLA and nucleus accumbens (199). This is not an exhaustive list of every GR manipulation model available but does highlight some of the keys models to help investigate body wide and cell specific impacts of GR manipulation. Despite numerous models for interrogating GR, no models thus far have been generated to investigate GR specifically in the adrenal cortex.

1.6.3 Interactions between AR and GR signalling

Androgens and glucocorticoids have a wide array of physiological targets and activities, but both receptors are very close members of the nuclear-receptor family and their mechanisms of action are extremely similar, both recognise very similar DNA-response elements and utilize overlapping co-regulators to mediate their effects on target genes. The primary separation of the effects of these receptors is their cell and tissue specific expression and the specificity of their respective ligands. However, in cell types that express both receptors, the mechanisms resulting in appropriate targeting are unclear (200). Various studies have shown that they are able to interact with each other in cellular processes. One of the most notable interactions has been in the development of prostate cancer. It has been hypothesised that GR activity in prostate cancer is dependent on the activation status of AR. GR has been shown to have anti-proliferative effects when AR signalling in not perturbed, however, blockage of androgen signalling has shown to increase GR expression and is thought to offer a by-pass survival mechanism and aid cancer progression (121, 201-203). This again was noted in bladder cancer where they suggested that AR drives pro-inflammatory response through interaction with isoform GR alpha (204). Additionally, studies have also identified that AR can negatively regulate GR transcription, and this is through a negative androgen-response element sequence near the GR gene (203). Studies have also suggested that the interaction between these two receptors is not purely limited in the context of disease progression, but is required during normal cellular signalling. The same group also showed that AR and GR form heterodimers, and the activation of hormone response elements is based on a co-operative process (205). Additional research would be required to investigate the full range of mechanisms and tissues that

AR and GR could potentially be working together in and what mechanisms they control.

1.7 The HPA Axis

Animals respond to environmental stressors by employing a number of physiological and behavioural responses, which are collectively termed the 'stress response'. Stress is universally described as perturbed homeostasis. To correct for stress, a number of process are involved including the endocrine, immune, and nervous systems. Behaviour and physiological response to stress serve the primary purpose of survival such as improved cognition, euphoria, increased respiratory rate, and cardiovascular tone and these are to name a few (206). Due to the wide array of physiological and effects of stress, a number of endocrine and neuronal systems work separately and in tandem to regulate the body's response to stress which is regulated by the hypothalamus-pituitary-adrenal (HPA) axis.

To summarise HPA axis feedback which will be described in detail in the sections below: stress is initially detected in the neurons localised in the paraventricular nucleus (PVN) of the hypothalamus, upon stimulation the PVN synthesizes and secretes corticotropin-releasing factor (CRF). Once released, CRF travels via the hypophysial portal vessels to target the anterior pituitary. CRF subsequently binds to its receptor on pituitary corticotropes and induces the release of ACTH, which is released into general circulation. Once in system circulation ACTH is able to target the adrenal cortex by binding to its receptor MC2R which is expressed in steroidogenic cells in the zona fasciculata. Upon binding of ACTH to MC2R, glucocorticoids are synthesised and released from the zona fasciculata. The regulation of the HPA-axis works on a negative feedback mechanism in which glucocorticoids bind to various regions throughout the brain to inhibit the stress response. However, this system can be perturbed and can result in a number of pathologies (207).

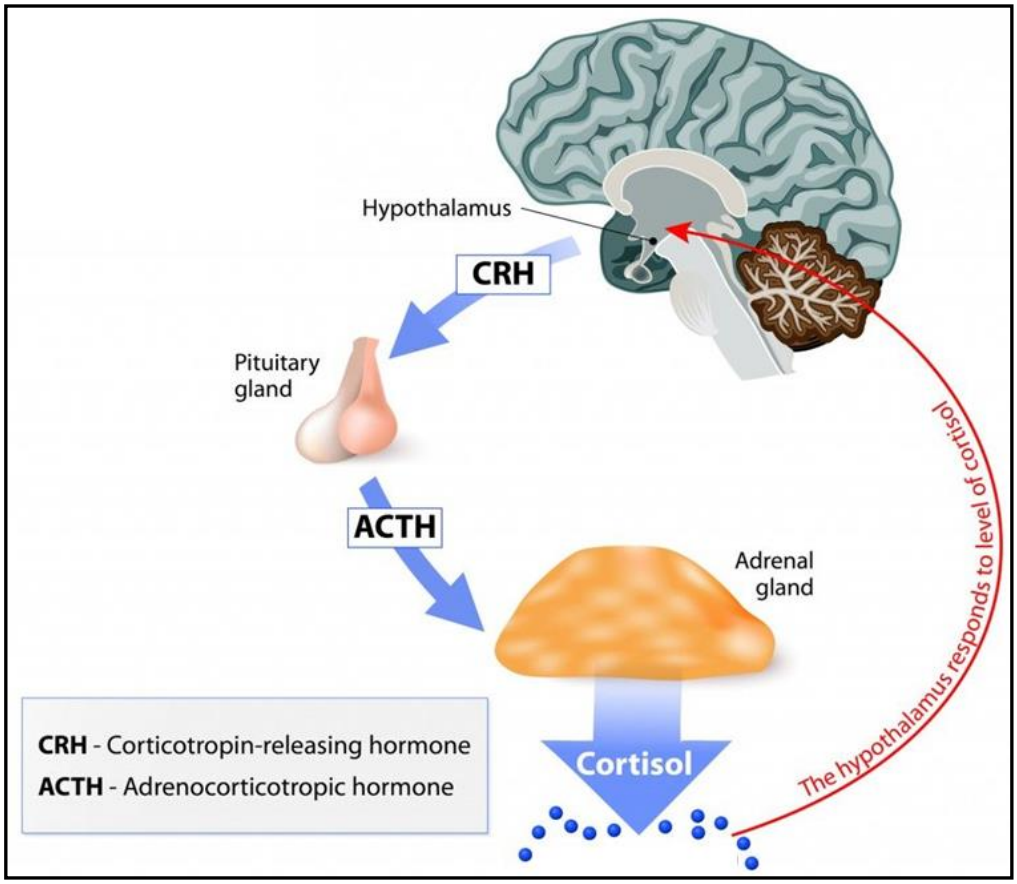


Figure 1-11. Diagram depicting basic HPA-regulation and feedback. *The hypothalamus produces corticotrophin-releasing hormone (CRH), which travels to the anterior pituitary. Here it stimulates the release of adrenocorticotropic hormone (ACTH). The release of ACTH into the bloodstream allows it to bind to the highly specific melanocortin 2 receptors (MC2R) on the surface of the zona glomerulosa, stimulating the production of cortisol. Cortisol acts at the level of the hypothalamus to produce a negative feedback loop and stop cortisol production. Adapted from (208).*

1.7.1 Anatomy of stress response

Appropriate regulation of glucocorticoid production and response is essential for survival, and instances resulting in too much or too little circulating can result in serious pathological conditions. Depicted in figure 1-11, a healthy response to stress sees a rapid increase in glucocorticoids following a stressful stimulus, this is shortly followed by a rapid decline and subsequent termination of the event. However, if the

stimuli is maintained in the case of chronic stress, this results in the prolonged exposure and body wide impacts of glucocorticoids catabolic properties, stress peptides and proinflamatory cytokines. This continued stimulus can result in neuropsychiatric, metabolic and endocrine disorders (209).

1.7.1.1 Components of the HPA axis

Corticotropin-releasing factor was originally isolated from ovine hypothalamic tissue in 1981, and has since been shown to be the primary regulator of ACTH release from the anterior pituitary corticotropes (210). The physiological actions of the CRF family of peptides exert their effects through two distinct receptor subtypes which belong to the class B family of G-protein coupled receptors, CRF type 1 receptor (CRFR1) and CRF type 2 receptor (CRFR2). The regulation of neuroendocrine activities by CRF are mediated through its receptor CRFR1 in anterior pituitary corticotrophs. CRF binds to CRFR1 and results in the stimulation of adenylate cyclase which is then able to stimulate the cAMP pathway and release of ACTH from the anterior pituitary into circulation. CRFR1's essential role in ACTH release was confirmed through experiments conducted in mice with an ablation of CRFR1, the results from this study demonstrated a highly disrupted HPA axis and increased anxiety related behaviours (211, 212).

The nonapeptide vasopressin is highly expressed in the PVN, supraoptic (SON), and suprachiasmatic nuclei of the hypothalamus. The parvocellular neurons of the PVN synthesize and release vasopressin into the hypothalamic portal circulation where it heightens the effects of CRF on ACTH release from the anterior pituitary. During periods of prolonged stress, the role of vasopressin becomes even more important as it maintains the responsiveness of ACTH to novel stressors (213, 214).

ACTH is formed from pro-opiomelanocortin (POMC), a prohormone that is highly expressed in the hypothalamus and pituitary. POMC is cleaved into a number of bioactive peptides, one of which is ACTH but also include β-lipotropic hormone, β-endorphin and melanocortins (215). Upon release from the pituitary, ACTH can now bind to its receptor MC2R in parenchymal cells of the zona fasciculata of the adrenal

cortex. The binding of ACTH to MC2R initiates the cAMP pathway that leads to the production of steroid products, such as glucocorticoids, mineralocorticoids and androgens (216). More specifically, ACTH initiates the conversion of cholesterol into 5-5 pregnenolone, an essential initial step in the production of glucocorticoids (8, 217).

1.7.2 Regulation of HPA axis

As described, the HPA axis is regulated via a negative feedback mechanism, under the control of glucocorticoids. Inhibition of the HPA axis occurs in the brain, however GR has been identified throughout numerous regions in the brain and determining the specific region of negative feedback remain elusive. However, two key regions have been identified, the hypophysiotropic neurons of the PVN and the hippocampus. The PVN was identified through experiments that examined the effects of locally administered glucocorticoids on the hypophysiotropic neurons of the PVN. This revealed that stimulation of these neurones was able to relieve the effects of adrenalectomy-induced ACTH hypersecretion. Again, experiments examining the impact of local glucocorticoid administration on the hippocampus revealed not only reduced stress response but also decreased basal glucocorticoids (218, 219). Glucocorticoids have also been shown to act at the level of the pituitary to modulate HPA activity and is thought to be the reason for the appreciable differences in HPA axis function across individuals (220). Two distinct mechanisms have been identified in which glucocorticoids can regulate this negative feedback through GR. The first and most well characterised mechanism occurs through a delayed process in response to circulating levels of glucocorticoids and works via genomic alterations (221). The second less characterised mechanism of feedback regulation works in response to the rate of glucocorticoid production and is thought to work via fast non-genomic actions, however this process is still not clearly understood (222).

1.7.3 Effects of chronic stimulation or under stimulation of the HPA axis

The stress response normally only lasts for a short period of time, however, during these short periods other bodily functions become supressed with no adverse impacts to the individual. These include the suppression of growth, reproductive systems, metabolism and immune response. Inappropriate activation of the HPA axis for longer than normal can lead to a number of disorders associated with chronic low or high levels of glucocorticoids, detailed in table 1-3 (223). Furthermore, disruption of signals to the adrenal can result in the alteration of cortex zones. Overstimulation signals or loss of signalling can result in enlargement or shrinkage of the affected cortex zone. This is not to be confused with the remodelling process that occurs during development in which there is a tightly regulated differentiation process (detailed in section 1.4), rather it is a loss or increase in the differentiated cell populations of the effected zones and the steroids they produce (224).

Increased HPA Axis	**Decreased HPA Axis**
Chronic stress	Adrenal insufficiency
Melancholic depression	Atypical seasonal/depression
Anorexia nervosa	Chronic fatigue
Obsessive-compulsive disorder	Fibromyalgia
Panic disorder	Hypothyroidism
Chronic active alcoholism	Nicotine withdrawal
Alcohol and narcotic withdrawal	Premenstrual tension syndrome
Panic disorder	Postpartum period
Diabetes mellitus	After chronic stress
Childhood sexual abuse	Menopause
Hyperthyroidism	
Cushing's syndrome	
Pregnancy	

Table 1-3. Summary of diseases associated with dysregulated HPA function. *Table highlighting the impact of sustained high or low circulating glucocorticoids.*

1.7.4 The HPG axis and its cross talk with the HPA axis

The hypothalamic-pituitary-gonadal (HPG) axis is essential for regulation of processes involved in development, reproduction and ageing, as well as many others. The regulation of the HPG axis is achieved through the release of gonadotropin releasing hormone (GnRH) from the hypothalamus which travels to the anterior pituitary to stimulate the release of luteinizing hormone (LH) and follicle stimulating hormone (FSH) into the blood stream in which it is able to target the gonads. In the testis, LH stimulates the leydig cells to produce androgens. In addition to this, FSH stimulates the sertoli cells to produce androgen binding protein (ABG) along with inhibin. The increased levels of androgens and inhibin then act to create a negative feedback at the pituitary and hypothalamus to stop androgen production. In the ovary, LH binds to theca cells and FSH binds to the granulosa cells to stimulate the production of oestrogens and inhibin. The increased levels of oestrogen and inhibin negatively regulates at the level of the hypothalamus (225). The HPG and HPA axis are not completely independent entities and research has highlighted cross-talk between the two feedback systems. One of the most notable examples of this is how the adrenal axis is able to regulate gonadal function. This is illustrated by the inhibitory effects of stress on reproductive behaviour and sex steroid release. Furthermore, several components of the gonadal axis can have reciprocal effects on the HPA axis. Studies investigating humans and rats noted that stress and high doses of glucocorticoids generally disrupt many aspects of the HPG axis including reproductive behaviour, LHRH expression, plasma LH secretion and sex steroid synthesis and release (226, 227). Conversely, oestrogens and androgens are able to act on several levels of the HPA axis, including adrenocorticoid synthesis, stress-induced ACTH and glucocorticoid release and CRH and AVP synthesis in the paraventricular nucleus (PVN) (109, 228).

1.7.5 Roles of AR and GR signalling in psychiatric disorders

Intense or chronic bouts of stress have been shown to result in emotional disturbances and hormonal disruption that can ultimately culminate in a psychiatric disorder (229). In patients that have been diagnosed with conditions such as depression or anxiety more than 50% of these have hyperactivity of the HPA axis (230, 231). HPA axis activation results in low affinity for food, low sex drive, increased blood flow to muscle, increased locomotive activity and raised blood sugars which prime the body to respond to a stress event (232). In normal conditions these bursts of activity last for only a few minutes at a time, however, prolonged stress is thought to overstimulate the HPA axis causing hypersecretion of CRH and can ultimately, if left untreated, lead to the dysregulation of the HPA axis potentially promoting the onset of a psychiatric disorder (233). Previous studies have highlighted the role of GR in the brain and its role in cognitive function (234). For example, it has been shown that glucocorticoids acting through GR in the hindbrain can enhance the arterial pressure in response to acute restraint stress (235) suggesting that GR is required for an appropriate stress response in healthy individuals. Persuasive evidence points to dysfunctional GR in HPA axis dysregulation, which has been successfully resolved upon antidepressant treatment (232). Additional transgenic studies that use antisense RNA complimentary to mRNA to knock down GR in the brain noted that mice display characteristics of depression in addition to altered behaviour (236). Using mouse models to mimic any human behaviour is an extremely difficult undertaking, especially in relation to psychiatric disorders which are often multifactorial. Patient symptoms are often heterogeneous in psychiatric disorders (237). For example, some patients experience excessive weight gain while other patients with the same psychiatric condition experiences weight loss or chronic fatigue and insomnia (238). In additional to this, a mouse's response to stress differs due to the lack of conscious self (239). However, stress behaviours that can be measured and quantified and therefore permit investigation of endocrinological and behavioural alterations have been identified in rodents that giving greater opportunity to better understand the aetiology of psychiatric disorders.

Androgens have interestingly also been suggested to play a role in the regulation of psychiatric conditions such as anxiety and depression. Men have been shown to have a decreased incidence of mood disorder diagnosis compared to women and this is thought to be due to the higher levels of circulating testosterone in men (240). This was also noted in a study that examined the salivary T levels in young girls and boys, and individuals that had lower testosterone experienced higher levels of anxiety and depression (241). Age related androgen decline has also been associated with negative mood and depression in aging men and women (242). Treatment with exogenous androgens in rodents also demonstrated improved cognition and performed better in anxiety behaviour tests, demonstrating increased explorative behaviour and time spent out in the open (243, 244). The mechanism underpinning the anti-anxiety effects of androgens were thought to be due to androgen signalling in the central nervous system (CNS), but cell specific ablation of CNS AR using Nestin-Cre demonstrated no impact on behaviour or the HPA axis and suggested this stress regulation was occurring at adrenal level (245).

1.8 Hypothesis

The proposed hypothesis is that androgen receptor and glucocorticoid receptor play an important role in the development and maintenance of the adrenal cortex and is essential in X-zone regulation and appropriate function of the HPA-axis.

1.8.1 Objectives

1. To utilize a Cre line previously developed in the Smith group to ablate androgen receptor or glucocorticoid receptor specifically from the mouse adrenal cortex.

2. To define the role of androgen receptor and glucocorticoid receptor in the regulation of the male adrenal cortex.

3. To define the role of androgen receptor and glucocorticoid receptor in the regulation of the female adrenal cortex.

1.8.2 Rationale

The regulation of the adrenal cortex by androgens was demonstrated as early as 1927 (246), and many androgen receptor ablation models have been developed from then in the form of global androgen receptor knockouts (160) and cell specific knockouts (163, 164, 245, 247), that have investigated various cell types in the brain and reproductive tissues. Despite this early identification of the potential impact of androgen signalling as a regulator of the developing adrenal, and numerous global knockout models targeting AR, very little work has touched upon the impact of adrenal AR signalling, especially in the rodent. This may be in part due to the fact that the rodent adrenal does not produce androgens and has therefore potentially been overlooked as a model to investigate its action (111). GR signalling has also been extensively researched and its resulting impact on the HPA axis (109), but this research has primarily focused on GR targeting in neuronal tissue (199, 248-250), thus far, no models have specifically ablated GR from the adrenal cortex. Furthermore, AR and GR have been shown to

interact in a number of cell types to control target genes (203, 205, 251, 252), but if they interact in the adrenal cortex has still not been described.

1.8.3 Experimental approach

The first steps in this project is to ablate AR or GR from tissues expressing a Cre targeted to the *Cyp11a1* locus that will permit the targeting of genes in steroidogenic cell types (253). In addition to gene ablation, further androgen manipulation will be achieved through castration, and hCG treatment. Following these methods, analysis will include, physiological, histological, cellular and molecular techniques to determine the underlying mechanisms involved in any potential phenotype that may arise.

Chapter 2. Materials and Methods

2.1 Mouse breeding and maintenance

2.1.1 Husbandry and Welfare

Mouse colonies were housed and maintained in the University of Edinburgh's BRF Little France Animal Facility. Mice were kept in strict housing conditions with humidity maintained consistently at 55%, ambient temperatures between 20-25 ºC. Mice were also kept in a 12 hour light/dark cycle. Food and water supply in the cages was unrestricted. Mice were maintained in adherence to conditions laid out by the Animals (Scientific Procedures) Act, 1986. Housing conditions and colony breeding adhered to ARRIVE guidelines (254) and UK Home Office regulations under project license 80/7704 held by Professor Lee Smith. Day to day animal husbandry and setting up of breeding pairs was carried out under my direction by Mike Dodds, whose help has been invaluable.

2.1.2 Timed Mating

To investigate embryos at specific time points, timed matings were performed. One male and one female were housed together in the afternoon, with copulation to be expected in the next dark cycle. Females were checked for vaginal plugs at 8am the following morning, this allowed appropriate calculation of gestational age of the embryos. Morning of plug discovery is designated e0.5. Mice are not sexually mature until postnatal day d50, so to ensure successful mating, mice from d50 onward were used.

2.1.3 Transgenic mouse lines; Cre/*loxP* recombinase

Cre recombination technology is a well described and extremely useful tool in investigating gene function within a specific tissue. This system works by method of site-specific recombination occurring in P1 bacteriophage (255) Cre recombinase is an enzyme that can recombine DNA between two 34 bp *loxP* sites. These sites consist of directional components that dictate the result of the recombination. If *loxP* sites are inverted this will result in an inversion of the DNA between these sites, if the directionality of *loxP* sites are repeated this will result in deletion of the DNA between

these two sites and if the *loxP* sites are located on different chromosomes, this can result in chromosomal translocation (256).

2.1.3.1 Cell-Specific Cre recombinase

Transgenes constructed with Cre recombinase driven by cell specific promoters permit the expression of Cre in specific cell types of interest. An additional level of control is achieved by introducing a fused Cre to a ligand-inducible nuclear translocation signal, such as that found in the nuclear hormone receptor family. A Cre generated using this method does not transport to the nucleus and execute recombination until introduced to a ligand specific to the hormone receptor of choice. A corresponding 'floxed' line is then generated to surround the selected sequence by *loxP* sites. The sequence selected can either be the whole gene, or part of a gene, such as an exon that codes for a region essential for that protein's function (257). The same mechanisms can also be used to investigate a 'reporter gene' which can be chromogenic or fluorescent. Transgenic constructs can be created with a reporter gene of interest inserted into a ubiquitously expressed locus with an upstream stop codon which is flanked by *loxP* sites. The resulting recombination permits excision of the stop codon and subsequent expression of the reporter gene. Setting up matings with a 'Cre' line to a 'floxed' line results in some offspring inheriting both transgenes and undergoing a cell specific ablation of the required gene, or the activation of a gene of interest. The general principle of this is illustrated in figure 2-1.

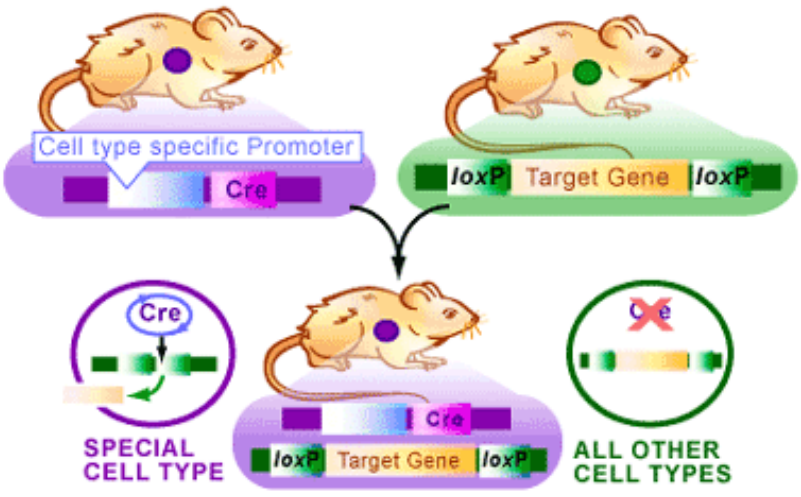


Figure 2-1. Cre/lox Mouse Breeding (258).

2.1.3.2 Generation of Cyp11a1-*GC Cre; YFP Reporter line*

This line was generated through use of Rosa26 mice (259) carrying an insertion of a yellow fluorescence protein (YFP) transgene downstream from a stop codon flanked by *loxP* sites (260). When females homozygous for Rosa26YFP (R26YFP) are bred to a male carrying a Cre transgene under control of a desired promotor, 100% of the resulting offspring will inherit one copy of the R26YFP transgene from the mother but 50% of the offspring will inherit the Cre transgene from the father, thus allowing cell specific expression of YFP (Figure 2-1). To generate adrenal specific knockout mice, Cre expression in the adrenal cortex needed to be confirmed, so R26YFP female mice were bred to males of a Cre line driven by a *Cyp11a1* promoter. This line was created by insertion of a GC-GFP transgene to the endogenous *Cyp11a1* locus (*Cyp11a1*$^{+/GC}$) (253). The *Cyp11a1*$^{+/GC}$ Cre line permitted cell specific recombinase action in tissues expressing *Cyp11a1* and thus an appropriate Cre line for conditional ablation of steroid genes from the adrenal cortex.

2.1.3.3 Generation of androgen receptor knockout mice

Ablation of androgen receptor specifically from the adrenal cortex was achieved through the use of the *Cyp11a1*$^{+/GC}$ Cre line as described in 2.1.3.1 and a floxed AR line. The AR floxed (AR^{flox}) line was generated through homologous recombination of a transgene construct to insert a *loxP* site in exon 1 and exon 3 of the AR gene (164, 247), thus resulting in a floxed exon 2 after recombination by Cre (261). Matings set up with females that were homozygous AR^{flox} to a male carrying a Cre of interest, will result in male offspring all carrying the AR^{flox} transgene. Due to the location of AR on the X-chromosome it is always inherited from the mother. Additionally, 50% of the litter will also carry the Cre transgene which will result in tissue specific recombination of AR. AR^{flox} females were bred to *Cyp11a1*$^{+/GC}$ males to produce offspring with total adrenal androgen receptor ablation (Ad-ARKO). As previously described, AR is on the X-chromosome and due to X-chromosome inactivation females from the aforementioned mating inherit a floxed AR from their mother but a wild-type AR from their father, resulting in mosaic adrenal AR ablation. To fully ablate AR from the adrenal cortex in females a second round of breeding is required, this involved the breeding on Cre+ Ad-ARKO male to an AR^{flox} female. The subsequent litters now

have females carrying complete ablation of AR from the adrenal cortex. Both generations were used in this thesis as part of the female study and were termed 1st Generation Ad-ARKOs and 2nd Generation Ad-ARKOs respectively. The AR^{flox} line used in this thesis was originally obtained from Karel de Gendt and Guido Verhoeven, University of Leuven, Belgium.

2.1.3.4 Generation of glucocorticoid receptor knockout mice

Ablation of glucocorticoid receptor from the hindbrain was achieved through the use of the *Cyp11a1*$^{+/GC}$ Cre line as described in 2.1.3.1 (253) and a floxed GR line. The GR floxed (GR^{flox}) line was generated through homologous recombination of a transgene construct to insert a *loxP* site into an EcoRI restriction site in intron 1-2 and upstream of a BamHI site in intron 3-4, thus resulting in floxed exon 3 (248). A *Cyp11a1*$^{+/GC}$ female was mated to a male homozygous for floxed GR The first generation from this mating resulted in litters heterozygous for GR^{flox} that were either Cre- or Cre+. For total GR ablation from the hindbrain, a Cre+ GR heterozygous male was mated to a GR^{flox} female. The resulting offspring were Cre- Hom termed 'littermate control' (LMC), Cre- Het, termed 'Het littermate control' (Het LMC), Cre+ Hom, termed 'Hindbrain GR knockout' (HB-GRKO), Cre+ Het, termed 'Het hindbrain GR knockout' (Het HB-GRKO). In addition to these mice, an external control group of c57bl/6J wildtype (WT) male and female mice that have had no interaction with GR knockout mice but have been housed on the same shelf and under the same conditions. These animals are termed 'WT Bl6'. GR^{flox} mice used in this thesis were obtained from an established colony being maintained in BRR little France animal facility, University of Edinburgh.

2.2 Treatments

2.2.1 Human chorionic gonadotropin (hCG)

It has been documented that hCG is able to stimulate Leydig cells to produce increased levels of circulating androgens. This treatment was used to generate mouse models with high circulating androgens. For single treatments, 200 IU/ml of hCG solution was IP injected at a volume of 0.1 ml to administer a final dose of 20 IU. Single injection

animals were culled 16 hours later. Injections were carried out at 5pm and collected the following morning at 9am. hCG treated animals. Injections were carried out by BRR technician Mike Dodds or by myself.

2.2.2 Castration

Generating mouse models with no circulating androgens was achieved through castration. This was achieved through making a single 1cm incision into the scrotum exposing the testes and removing them. Following removal of the testes, the site of incision was closed with sterile sutures. Mice were injected subcutaneously with Buprenorphine 0.05mg/kg whilst anaesthetised, then were allowed to recover whilst being monitored. Mice were then closely monitored over the next 24 hours for any welfare problems, and twice daily from then onwards. Castrations were carried out by BRR technician William Mungal. Mice were then collected two weeks later to ensure complete removal of any circulating androgens, or in long term castrations, 12 weeks later.

2.3 Necroscopy

2.3.1 Culling of Mice

Culling of postnatal animals was carried out by inhalation of rising CO_2 concentrations. Confirmation of death via cervical dislocation. In instances when bloods or pituitaries were collected, animals were culled via inhalation of CO_2 and once bloods were collected confirmation of death was carried out by exsanguination through cutting the main artery in the leg or underarm. Culling of neonates was performed by decapitation.

2.3.2 Whole mouse observations and measurements

After culling, mice were examined for overall condition which included coat condition and any signs of barbering; as the adrenal was targeted this can be a clear indication of stress. As androgens were disrupted each mouse was also weighed and anogenital distance (AGD) measured through the use of electronic callipers (Faithful Tools, Kent, UK). AGD is a standard measurement for androgen action throughout development (262).

2.3.3 Collection of bloods and serum

To analyse circulating hormones, bloods were collected immediately after CO_2 inhalation via cardiac puncture. Due to the quick nature in which serum levels of ACTH and corticosterone can rise due to handling stress, animal cage movement and handling was kept to a minimum prior to culling. All blood samples were taken at 10am. A 21 gauge needle (Becton Dickson, UK) was used with a wide bevel to prevent blood lysis, collected into a 1ml syringe (Becton Dickson, UK). The needle was not coated with an anticoagulant but following collection blood samples were immediately decanted into 0.5% EDTA coated tubes (Greiner bio-one, Austria). Due to the unstable nature of adrenocorticotropic hormone (ACTH) blood needed to be centrifuged within 20 minutes of collection. The blood samples were centrifuged at 26,054g for 10 minutes in a 4^{o}C chilled microcentrifuge. Centrifugation resulted in two the formation of two layers: serum and pellet. Serum should be clear, if samples are pink or red this denotes red blood cell lysis and could impact serum quality. Serum was then carefully collected and stored at -80 ^{o}C for long-term storage.

2.3.3.1 Serum analysis by ELISA

Analysis for the presence of corticosterone (Abora Assays, KO14-H5) or ACTH (Antibodies Online, ABIN415571) in serum samples was achieved through enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions. This assay is able to measure the concentration of an antigen in a sample of interest by binding to a target antibody. There are a few variations of ELISA kits, the two methods that were used in this thesis were a sandwich ELISA for corticosterone and a competitive ELISA for ACTH. A sandwich ELISA works by quantifying antigens between two layers of antibodies termed 'capture' and 'detection' antibodies. The general principle for this method is as follows: Plate is coated with capture antibody; the sample is then added to the plate and any antigen binds to the capture antibody; the detection antibody is added and binds to the antigen; enzyme-linked secondary antibody is added and this then binds to the detecting antibody; finally, the substrate is added and this converts the enzyme into a detectable form which can then be analysed. A competitive ELISA works based on a competitive-binding process,

achieved through a sample antigen and an add-in antigen. The general principle for this method is as follows; an unlabelled primary antibody is incubated with a sample antigen; these complexes are then added to a 96-well plate which are pre-coated with the same antigen; washes are then performed to remove any unbound antibody remaining (the more antigen in a sample of interest will result in less antibody able to bind to antigens in the plate); the secondary antibody that is specific to the primary antibody and conjugated with an enzyme is then added; a substrate is then added that reacts with enzymes to elicit a colour change inversely proportional to the amount of antigen in a sample. Once this is done, plates can be analysed on a plate reader at the manufacturers recommended wavelength. Both Assay were analysed on a plate reader at a wavelength of 450nm. Plates were analysed using ELISAanalysis.com to plot a standard curve. The standard curve was created with the log of ACTH/Corticosterone concentration on the y-axis and absorbance on the x-axis. The best fit straight line through the standard points was determined by regression analysis.

2.3.4 Collection of Tissues

Organs of interest were collected as described in the subsequent sections. Tissue was either frozen immediately in dry ice for RNA analysis or fixed in Bouins (Clin-Tech, Guildford, UK) for histological analysis.

2.3.4.1 Adrenal tissue

To prevent RNA degradation, tissues were collected immediately after culling. To recover adrenals, access to the main cavity was required. This was achieved through an incision made in the stomach following a wash with 70% ethanol above the genitals to avoid disruption and an incision made on both sides of the body. As the adrenals are located on top of the kidney, the intestines had to be carefully moved to one side. To provide experimental consistency, the left adrenal was collected and fixed in Bouin's solution for four hours. The right adrenal was more difficult to collect as it is located behind the intestine and liver and is closer to a main artery compared to the left adrenal, this on occasion lead to disruption or damage during collection and for these reasons the right adrenal was used for RNA analysis as the tissue would ultimately be homogenised. Adrenal tissue for RNA analysis was snap frozen in dry ice and stored

at -80^{o}C long-term. Adrenals are surrounded by a lot of fat, for removal, a fat pad was lifted with fine dissection forceps and a cut made between the adrenal and the kidney, with careful attention made to not grab the adrenal itself. Once removed from the body, the adrenal still had a lot of fat surrounding it. For accurate weight measurement and to prevent contamination of future RNA samples fat was removed with fine dissection scissors ether by eye or under a dissection microscope. As it has been previously noted that the adrenals on the left and right have slightly different shapes, a small cohort (n=5) were collected were the right adrenal was fixed and the left adrenal frozen. Human adult adrenal tissue was generously gifted by Dr Anne Jorgenson of Copenhagen University, Denmark. Two individual male samples were provided following patient biopsies. Foetal adrenal tissue was generously gifted by Dr Rod Mitchell, Edinburgh University, UK. Again, two individual male samples at 19 weeks, were provided from human foetal tissue obtained via elective abortion. Ethical approval for human tissue was obtained for the use of archived human testicular tissue from the pathology departments at the Western General Hospital in Edinburgh (REC Reference number-10/S1402/33) and Erasmus MC-University Medical Centre, Rotterdam (Institutional review board-MEC 02.981 and CCR2041).

2.3.4.2 Testis tissue

Once the abdomen was opened as described in 2.3.3.1, the testis were pulled up from the scrotum by using forceps to pull on the fat pads. This results in the movement of the testis from the scrotum to the abdomen for easy removal. Again, handling of the testis was by holding the surrounding fat to avoid organ damage. Only testis tissue was collected, therefor careful removal of the epididymis and vas deferens was performed. Once removed, the testes were weighed and left testis was fixed in Bouin's for 5 hours, cut in half and left to fix for another hour to ensure complete penetration of fixative. The right testis was cut in half and snap frozen in dry ice then kept at -80^{o}C for long-term storage.

2.3.4.3 Ovary tissue

The main body cavity was opened as described in 2.3.3.1. Intestines were carefully moved to the side to expose the ovary and the uterine horn. The uterine horn was followed to the top to locate the ovary. The ovary was lifted by surrounding fat and a

cut made between the ovary and the uterine horn and any remaining fat removed. For consistency the left ovary was fixed in Bouin's for four hours and the right ovary was snap frozen in dry ice and kept in -80 for long-term storage.

2.3.4.4 Brain and pituitary tissue

To collect brain and pituitary tissues, the head was first washed with 70% ethanol, mice where then decapitated carefully to ensure no disruption to the base of the skull to protect the pituitary. Hair was then removed and in incision was wade from the back of the skull to between the eyes, scissors were then delicately placed between the incisions and opened. This resulted access to the brain, which was then delicately lifted out and dissected into the fore, mid and hindbrain sections. These sections were individually snap frozen in dry ice and stored at -80 for future genomic DNA extraction. Once the brain was removed the pituitary could then be seen at the base of the cranium. The pituitary was carefully removed by placing fine forceps underneath the pituitary and scooping it out. Pituitaries used in this study were all snap frozen in dry ice and kept at -80 for long-term storage.

2.3.4.5 Foetal adrenal

To obtain embryos, plug checked pregnant dams were culled via inhalation of CO_2 and confirmation of death via cervical dislocation. The pregnant dam was then opened as described in 2.3.3.1 to reveal the pregnant uterus. The whole uterus was removed and then each embryo was removed from its amniotic sac and placed immediately into chilled PBS if fine dissection was required or into Bouin's fixative for 8 hours. Fine dissection of adrenals were then placed into Bouin's for 2 hours.

2.3.4.6 Analysis of tissue weights

To be able to appropriately analyse organ weight, for each experimental group an n=5-8 were averaged and plotted using Prism GraphPad 7. Error bars displayed on graphs are standard error of the mean (SEM) and were analysed using the most appropriate statistical test depending on ages being analysed or number of groups being compared.

2.3.4.7 Barbering Scale

To determine if barbering observed falls out with normal cage behaviour, hair loss was graded as follows; No hair loss = 0, removal of whiskers = 1, removal of whiskers and facial hair = 2, removal of whiskers, facial hair that extends to head = 3, removal of whiskers and hair on the face, head and back = 4 and removal of whiskers and hair on the face, head, back and stomach = 5. Hair loss that was noted from 0-2 was deemed to fall within the normal range of barbering that would be expected in WT animals, any barbering scored 3 or higher was deemed abnormal and was more severe than would be expected under normal conditions (263, 264).

2.4 Histology

2.4.1 Fixation, embedding and sectioning of tissues

After being fixed in Bouin's for the appropriate amount of time, tissues were removed and then placed into 70% ethanol for storage (no more than 6 months) until they were processed for embedding. Samples undergo automated processing which involves Bouin's fixed tissue placed through a series of graded alcohols for 17.5 hours in an automated Leica TP1050 tissue processor (Leica Microsystems, Milton Keynes, UK) and embedded by hand in paraffin wax. Tissue processing was carried out by Garry Menzies in the SURF department of University of Edinburgh.

To be able to perform analysis on embedded tissue, a microtome (Leica, model RM 2135) was used to cut tissue section with a width of 5µm. These sections were then floated in a water bath (Lamb RA, model E/65) to help dissipate any wrinkles in the wax before mounting on slides that were statically charged to provide appropriate adherence of the tissue to the slide. For health and safety purposes, white slides were used for mouse tissue and pink slides for human tissue (Leica Biosystems, Germany). Slides with mounted sections were then dried overnight in an oven set to 50 ^{o}C. Slides were allowed to cool before performing any analysis.

2.4.2 Haematoxylin and Eosin (H&E) staining

To be able to perform histological analysis tissue sections need to be stained to make tissue structures visible which is made possible through the use H&E staining. Slides are first dewaxed, this is achieved by immersion in xylene for 5 minutes, and this step

is performed twice. Following xylene, slides are then rehydrated which involves immersing the sections in decreasing concentrations of alcohol (figure. 2-2). After briefly being washed with tap water tissue is then stained for 5 minutes in haematoxylin, this stains the nuclei blue. Slides are again washed in tap water and placed in 1% alcohol acid, again briefly washed in tap water and placed into Scott's tap water, eosin for 30 seconds, tap water, and then dehydrated by increasing concentrations of ethanol. Slides were finally placed in xylene for 5 minutes and then mounted with a coverslip (VWR, UK) and Pertex mountant (Cell path, UK).

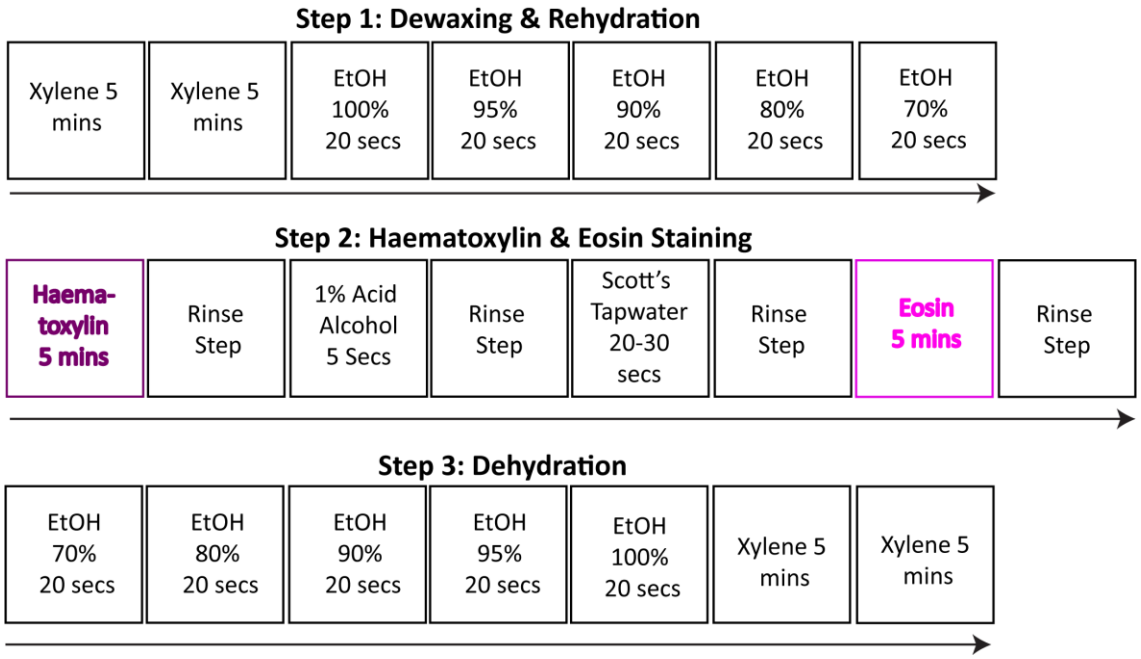


Figure 2-2. Schematic of H&E protocol

2.5 Immunohistochemistry

Immunohistochemistry is an extremely useful tool that permits the visualization of a specific protein in thin sections of fixed tissues. This can be achieved either via chromogenic or fluorescent detection systems that use an antibody against the protein of interest. Visualization of proteins in experimental animals was performed on no fewer than five different samples per group. All immunohistochemistry experiments performed included appropriate littermate and age matched controls, in addition to a

positive control slide, which the antibody in known to work in, along with a negative control to ensure staining observed is not unspecific or inappropriate binding.

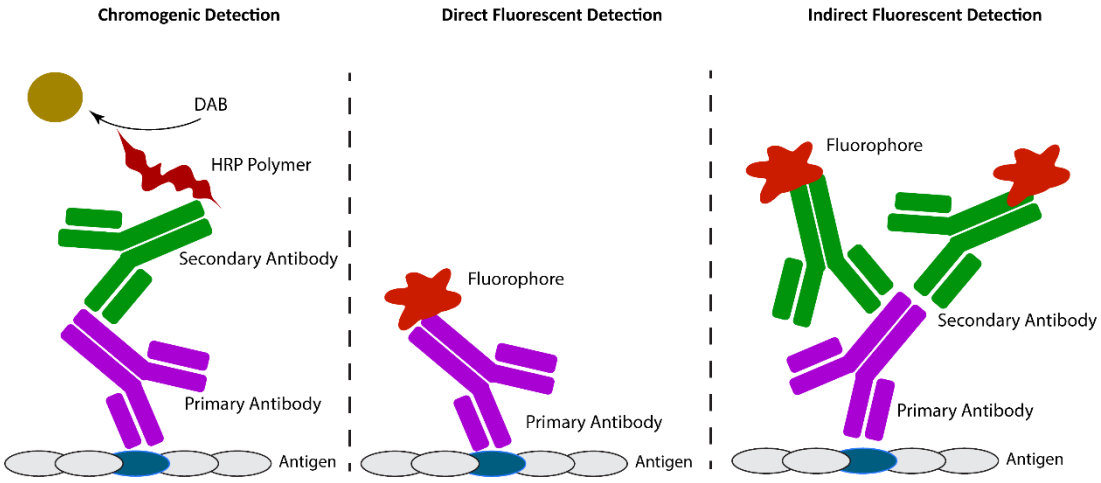


Figure 2-3 Schematic of different immunohistochemistry techniques

2.5.1 General protocol

Detailed steps are denoted in subsequent sections (2.5.1-2.5.9)

- Section fixed in Bouin's and embedded in paraffin wax were sectioned at 5µm and placed on a slide and dried.
- Sections went through dewaxing and rehydration.
- Slides were placed in a decloaking chamber for antigen retrieval
- Non-specific antigens were blocked through use of serum
- Tissue was then incubated at 4^0C overnight in specific primary antibody
- A secondary antibody was then added and detected using various amplification systems

- Protein of interest was detected using various colour detections. This could be achieved through colour reaction (DAB) or fluorophore excitation (direct or indirect fluorescence)
- Once stained slides were then covered with a coverslip with pertex or Permafluor depending on method used.

2.5.2 Sectioning, dewaxing and rehydrating

These steps are detailed in sections 2.4.1 and 2.4.2

2.5.3 Antigen retrieval

Tissue fixatives can impact the ability for a primary antibody detect antigens of interest. This occurs in Bouin's fixative and this is because it creates protein cross-linking which is very beneficial in maintaining the integrity of tissue samples but this results in masking of protein and antigenic sites. For best results and best antigen detection, cross link removal was needed. This was achieved in a pressure cooker (Instapot, Amazon, UK) which enabled heat induced antigen retrieval. Slides were rehydrated, placed in a plastic rack and immersed in a citrate buffer solution. Citrate buffer pH depended on the antibody being used, concentrations commonly used were pH6 and pH9 (25mls citrate buffer; and 225mls of dH_2O). Following immersion of slides in citrate solution they were placed in the pressure cooker for a 50 minute cycle. Temperature increased over a 20 minute period to 125 ^{o}C and was maintained for five minutes. At the end of the five minute period the pressure cooker was vented and allowed to cool for 20 minutes before removal. Slides were cooled and rinsed in tris buffered saline (TBS; Section 2.12.2.1) for five minutes.

2.5.4 Blocking non-specific sites

Antibodies will show preferential avidity for specific antigens, however there is the potential for partial non-specific binding to sites on non-specific targets particularly when polyclonal primary antibodies are used. Preventing non-specific binding is essential as it results in background and has the potential to mask the antigen of interest. Prevention of non-specific staining can be achieved through peroxidase

blocking and serum blocking. Peroxidase blocking is essential for blocking endogenous peroxidases found in the tissue, if not blocked, can result in high background and unspecific staining. This blocking step is particularly important in human tissue, however is also important for some rodent tissue. To do this, slides are first washed in hydrogen peroxide (25mls) and TBS (225mls) to produce an 3% H_2O_2;1x TBS solution. Slides were submerged in this solution for 15 minutes minimum to block endogenous peroxidases. Concentrations of hydrogen peroxide solutions can vary depending on type and species of the tissue and must be checked before completing this step. Higher concentrations of endogenous peroxidases need a stronger hydrogen peroxide concentration when washing. After this step slides were then washed in 1x TBS for 2x 5 minutes. Following these washes, slides were dried and tissue drawn round with a hydrophobic pen (Vector, UK). Preventing non-specific binding is essential as it results in background and has the potential to mask the antigen of interest. Prevention of non-specific staining can be achieved through serum blocking. Diluted blocking serum, described in section 2.12.1.6 was chosen based on the species the secondary antibody was raised in. Slides were then incubated for 1 hour in a humidity chamber at room temperature.

2.5.5 Primary antibody

Before use on experimental tissue each primary antibody was optimised for all samples and ages examined in this thesis. The primary was diluted in the specific serum and evenly distributed over the tissue. A negative control was included which had no primary antibody and serum only and incubated at 4^{o}C overnight in a humidity chamber. Primary antibodies used and dilutions are detailed in the table below (Table 2.1).

Protein stained for	Method	Dilution	Primary antibodies
HSD3B	Single fluorescence	1:1000	HSD3B: Santa Cruz #sc30820
AR	Single fluorescence	1:800	AR: Spring Bioscience #M4070
AR-N20	DAB	1:100	Santa Cruz Biotechnology #sc-816
PCNA	DAB	1:1000	Sigma #P-8825
AKR1B7	Single fluorescence	1:25	Santa Cruz Biotechnology #sc-27763
HSD20alpha	Single fluorescence	1:1200	Aviva Systems Biology #40002
GR	DAB	1:200	Cell Signaling #12041
CYP21	DAB	1:100	Bioss antibodies #bs-2443R
CASP3	Bond	1:100	CASP3: Abcam #ab4051

Table 2.1 Primary antibodies, method and dilutions for immunohistochemistry

2.5.6 Secondary antibodies

To remove primary antibody slides were washed in 1x Tris-Buffered Saline-triton (TBST) for 1x 5 minutes and 1x TBS for 2x 5 minutes. Following these washes the secondary was added to the tissue. All secondary antibodies used are described below in table 2.2.

2.5.6.1 DAB chromogenic secondary antibody protocol

Following the wash steps in section 2.5.6, slides were wiped to remove any excess buffer before applying a biotinylated secondary antibody diluted in appropriate serum. Slides were then left to incubate for 30 minutes in a humidity chamber at room temperature. Slides were then washed as previously described. Once wiped clean, slides were then treated with streptavidin HRP reagent (Dako, UK) diluted 1:1000 in 1x TBS at room temperature for 30 minutes in a humidity chamber.

2.5.6.2 Fluorescent secondary antibody protocol

Washes to remove primary antibody were performed as described 2.5.6. Slides were wiped clean to remove any excess buffer before applying the secondary. Depending on the primary antibody, the appropriate peroxidase-conjugated secondary antibody was applied diluted at 1:200 in appropriate serum and incubated for 30 minutes in a humidity chamber at room temperature.

2.5.7 Antigen detection

2.5.7.1 DAB Chromogenic detection and counterstain

Amplification of the biotinylated secondary antibody signal was achieved through incubation with streptavidin/horseradish peroxidase (HRP) (Vector, UK). Streptavidin was diluted 1:500 in 1xTBS in a humidity chamber for 30 minutes at room temperature. To visualise the antibody, slides were then incubated in 3, 3'–diaminobenzidine (DAB; DAKO) diluted 1 drop/ml of supplied buffer and used immediately. A successful reaction resulted in the development of a brown pigment located at antigenic site of interest as monitored under a microscope. This is a result of streptavidin catalysing the deposition of the brown pigment (Fig 2-3). Once a brown pigment could be observed the reaction was stopped by emersion in water. Slides were then counterstained for 5 minutes with haematoxylin.

Secondary antibody	Conjugate	Dilution	Product number
Chicken anti Rabbit	Biotinylated	1:500	Santa Cruz Biotechnology (sc-2986)
Goat anti Rabbit	Biotinylated	1:500	Vector, UK (BA-1000)
Goat anti Rabbit	Peroxidase	1:200	Vector, UK (PI-1000)
Rabbit anti Goat	Peroxidase	1:200	Sigma (A5420)
Donkey anti Goat	Peroxidase	1:200	Santa Cruz Biotechnology (sc-2020)
Mouse on Mouse HRP Polymer	Peroxidase	Neat	Abcam (ab-127055)

Table 2.2. Secondary antibodies and dilutions

2.5.7.2 Fluorescence detection and counterstain

For visualization of peroxidase labelled secondary antibodies, slides were incubated for 10 minutes in a fluorescent dye using the 'Tyramide Signal Amplification' system (Perkin Elmer, USA) at room temperature in a humidity chamber. Detection of tyrimide works through HRP catalysing the deposition of fluorescent precipitate on the tissue. Tyramide was diluted 1:50 in supplied buffer and used immediately. After a ten minute period the reaction was stopped by immersing slides in 1x TBS for 3x 5 minute washes. Once tyramide was applied and washed off slides were kept covered as much as possible to prevent bleaching of the tissue. Following TBS washes, sections were then stained in Sytox green (Invitrogen, USA) diluted 1:12000 for ten minutes in a humidity chamber and ensuring minimal exposure to light. Following this, the slides were washed in 1xTBS for the last time.

2.5.8 Dehydration and mounting

For DAB staining, once slides had been counterstained with haematoxylin they were rehydrated as per the protocol described in 2.4.2. Once finished in xylene slides were moved into mounting xylene and covered with glass coverslips, adherence of coverslips was achieved by the use of pertex (Cell path, UK) and allowed to dry for 2 hours and can be imaged or stored at room temperature. Mounting slides for IF does not require dehydrating, coverslips are adhered to the slide through the use of Permafluor (Thermo scientific). Slides were allowed to dry for a minimum of 4-5 hours and could be imaged or stored at 4^{o}C for up to six months.

2.5.9 Automated immunohistochemistry for Cleaved Caspase 3

Another method used for antigen detection can be achieved through the use of an automated immunostaining machine (Leica Microsystems, UK) termed 'Bond'. This automated machine can do up to 30 slides per run, with the use of a polymer amplification instead of streptavidin. For experimental cohorts requiring cleaved caspase staining automated immunohistochemistry was performed using cleaved caspase primary antibody (Cell Signalling Technology) diluted 1:100. This dilution was chosen based on an optimisation run with appropriate tissue and controls. A Polymer Refine Detection kit according to standard manufacturer protocol. Slide

mounting was also automated. Slides could then be stored at room temperature. This protocol was optimised and run by Lyndsey Boswell SURF, University of Edinburgh.

2.5.10 Microscopy

2.5.10.1 Light Microscopy

Slides stained using DAB or Bond immunodetection were examined using a Provis microscope (Olympus Optical, London, UK) and images were captured on a digital camera (Canon DS126161) attached to the microscope. Images were also captured on an Axiovert Scan.Z1 slide scanner (CarlZiess, Germany). These enabled automated scans of whole tissue sections. Images captured by both methods were captured edited on Zen lite 2 software.

2.5.10.2 Fluorescent Microscopy

To visualise immunostained sections, slides were imaged on a Ziess LSM 710 or LSM 510 Axio-Imager microscope (CarlZeiss Ltd, UK). This microscope was fitted with an HVC20 camera (Hitachi Denshi Europe, UK). Images were also captured on an Axiovert Scan.Z1 slide scanner (CarlZiess, Germany). These enabled automated scans of whole tissue sections. Images captured by both methods were captured edited on Zen lite 2 software.

2.6 Stereology

Stereology is an important tool in providing interpretation of a two-dimensional cross section of tissue. It provides the ability to extract quantitative data about a three dimensional tissue from a measurement taken on a two-dimensional section. Providing measurements like these helps confirm visual morphology observations. H&E stained slides were examined using a Provis microscope (Olympus Optical, London, UK) and images were captured on a digital camera (Canon DS126161) attached to the microscope. Images were also captured on an Axiovert Scan.Z1 slide scanner (CarlZiess, Germany). These enabled automated scans of whole tissue sections. Images captured by both methods were captured edited and measured on Zen lite 2 software.

2.6.1 Serial Sectioning for cortex measurements

Due to the unusual shape of the adrenal, measurements taken at random sections throughout the adrenal would give inconclusive and highly variable results between sections and experimental cohorts. To account for this variation in shape, adrenals were serial sectioned the whole way through with one section per slide. One adrenal provided on average 40-50 single section slides. For H&E staining and analysis, slides 15, 25 and 35 were selected from each adrenal. This provided a good range throughout the adrenal that had the presence of all cortex zones. Each group analysed had an n=5.

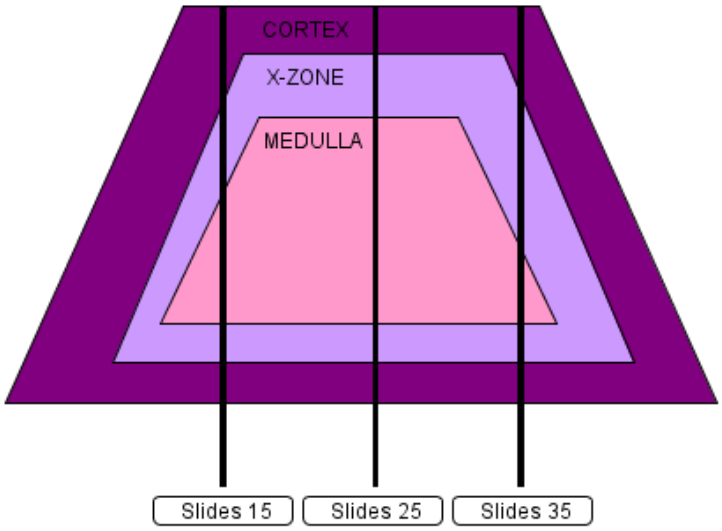


Figure 2-4 Schematic detailing regions slides were taken from to perform cortex measurements.

2.6.2 X-zone cell density measurements

Cell density of X-zone cells were counted at x40 magnification. The image once opened had a 5x4 square grid placed over the top of the image. Each square was assigned a number 1-20 and 5 of these sections were randomly selected with a calculator. Once the grids were selected, an event marker was used to count the number of cells present in each 2000 µm square. Cells were counted in a similar fashion to cell counts on a haemocytometer, cell touching the bottom and right light line were included in the box, cell touching the top and left sides of the box were not. These measurements were then recorded under the measurement tab in the Zen profile and could then be exported into excel. The image could be saved with all these measurements so they can be accessed after the image file has been closed (Fig. 2-5).

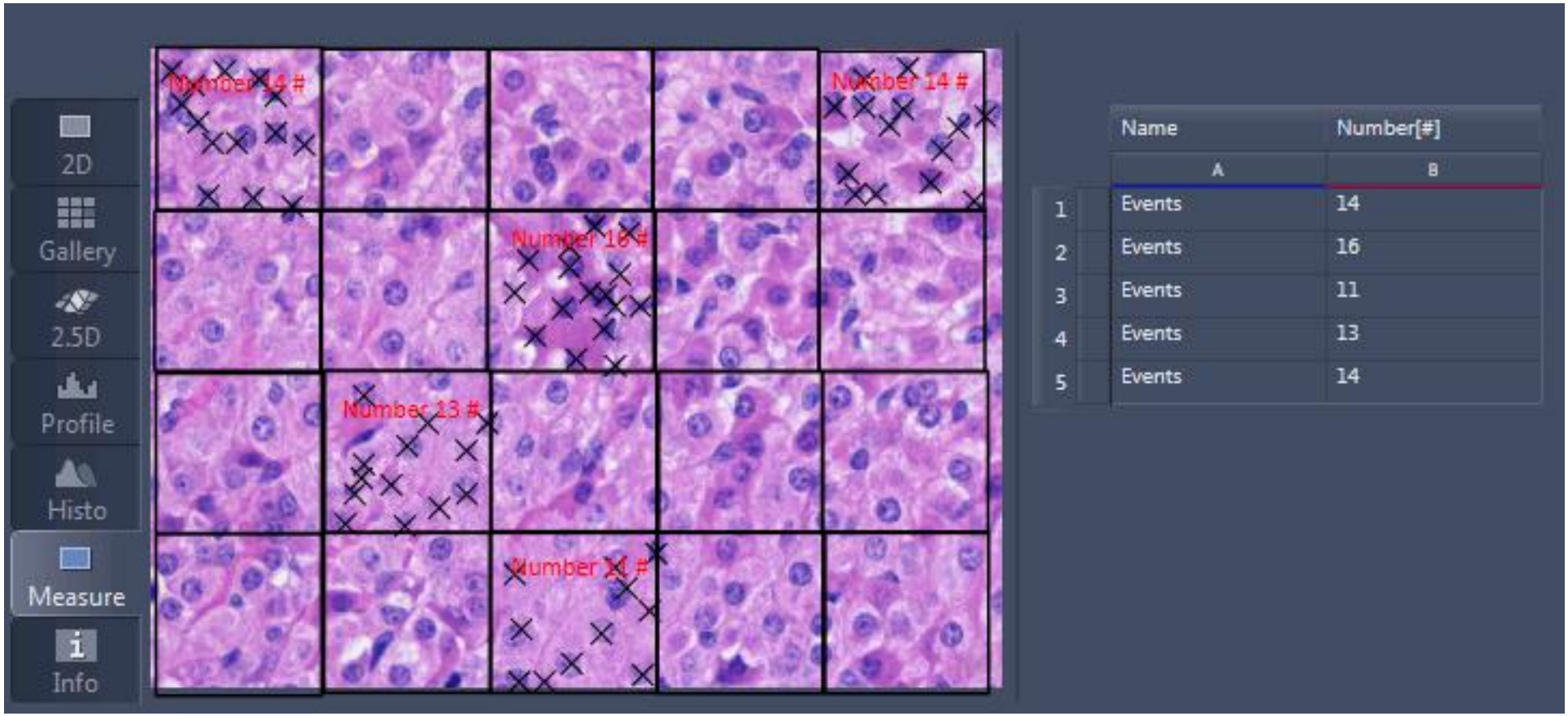


Figure 2-5. Screen grab of X-zone cell density measurements in Zen lite 2. *An image of an X-zone at x40 magnification. Numbers for individual square counts can be seen on the right under name and number.*

2.6.2 X-zone depth measurements

To measure the proportion of the cortex the X-zone was occupying, the distance of the X-zone from the cortex medulla boundary to the outer edge of the X-zone was measured on x10 magnification images. Five individual measurements were taken at even intervals along the X-zone, these measurements can be seen figure 2-6.

2.6.3 Whole cortex depth measurements

The thickness of the whole adrenal cortex was measured at x10 magnification from the cortex-medulla boundary to the outer cortex. These distance measurements were taken at even intervals along the cortex. These measurements can be seen in figure 2-7.

2.6.4 Combined zona glomerulosa and zona fasciculata measurements

To determine if presence of an X-zone impacted the remaining adrenal cortex size, combined measurements of the zona glomerulosa and zona fasciculata thickness were calculated from subtracting the X-zone depth from the overall cortex depth.

2.6.5 Spindle cell occurrence

Analysis of spindle cell occurrence was achieved via serial sectioning 5 samples per experimental group. Multiple sections were selected evenly throughout each block and H&E stained to carry out morphology analysis. When the presence of spindle cells were observed it was noted and converted into a percentage out of 100. For example, 5 samples analyzed if only one sample presented with spindle cells it would equate to 20% occurrence for that group. These measurements accounted for presence/absence not severity.

2.6.6 PCNA positive cell counts

To investigate proliferation in control and experimental groups, sections were stained for PCNA under the protocol described in 2.5. Five sections from each sample were stained with an n=5 per group. Each section was scanned on the Axioscan to provide a whole section view. These sections were then counted for every PCNA positive cell observed in that section in Zen lite 2, these counts can be seen in figure 2-8.

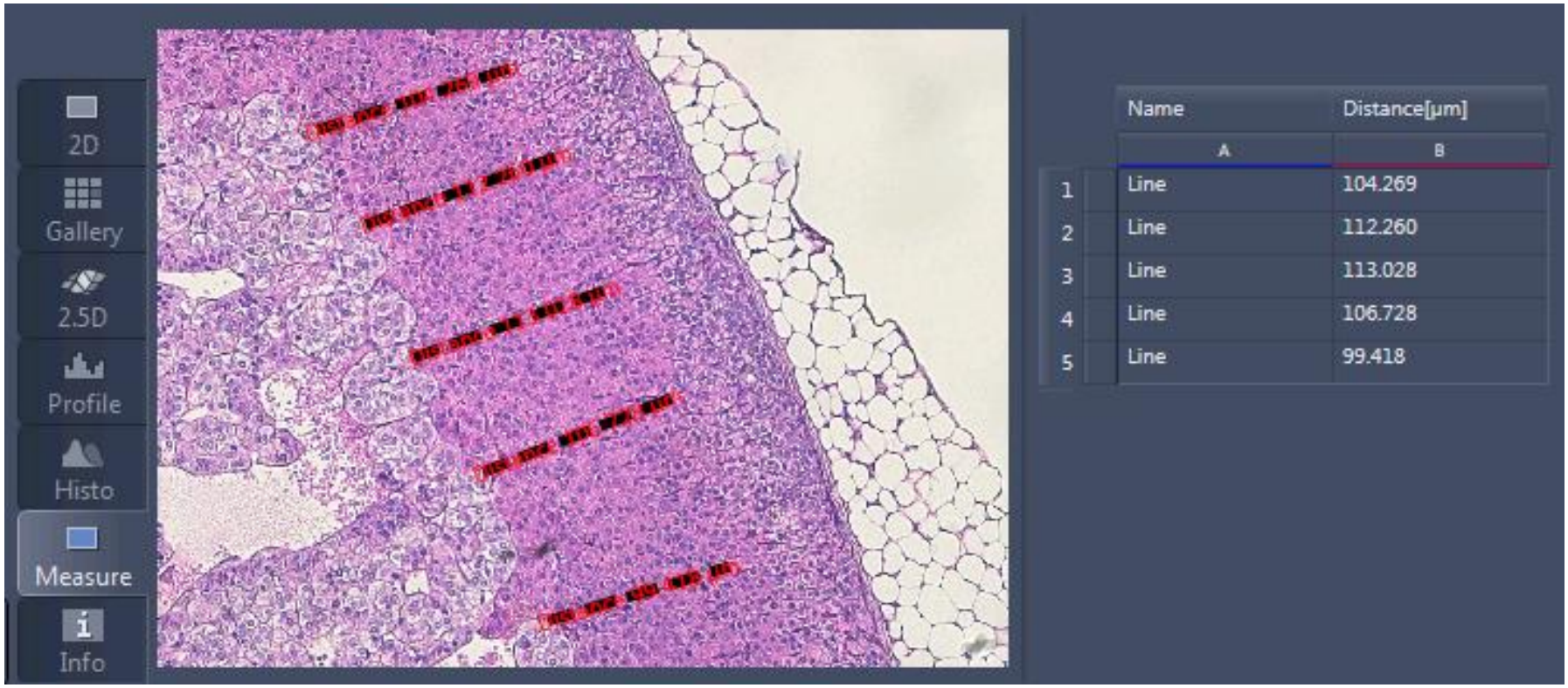


Figure 2-6. Screen grab of X-zone depth measurements in Zen lite 2. *An image of an X-zone at x10 magnification. Numbers for individual line measurements can be seen on the right under name and number*

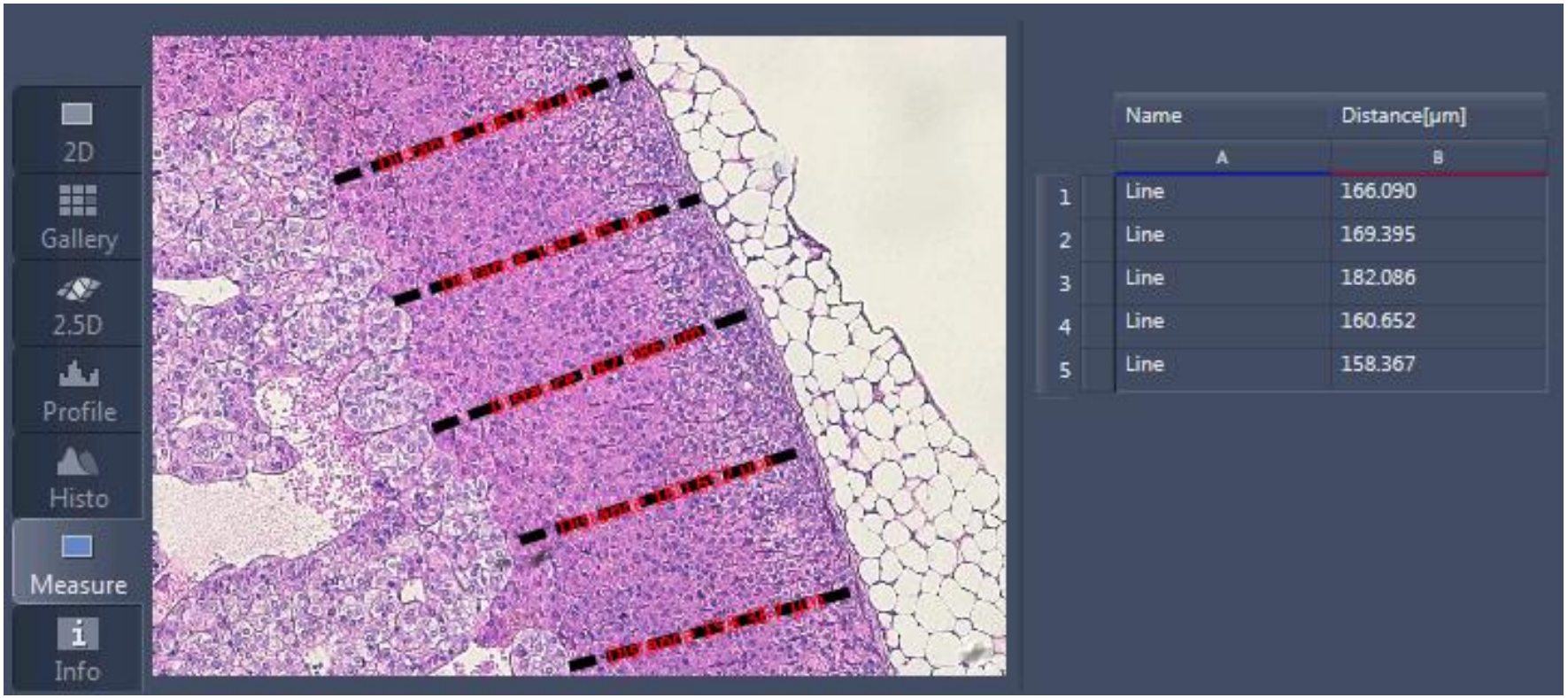


Figure 2-7. Screen grab of whole cortex depth measurements in Zen lite 2. *An image of an adrenal at x10 magnification. Numbers for individual line measurements can be seen on the right under name and number.*

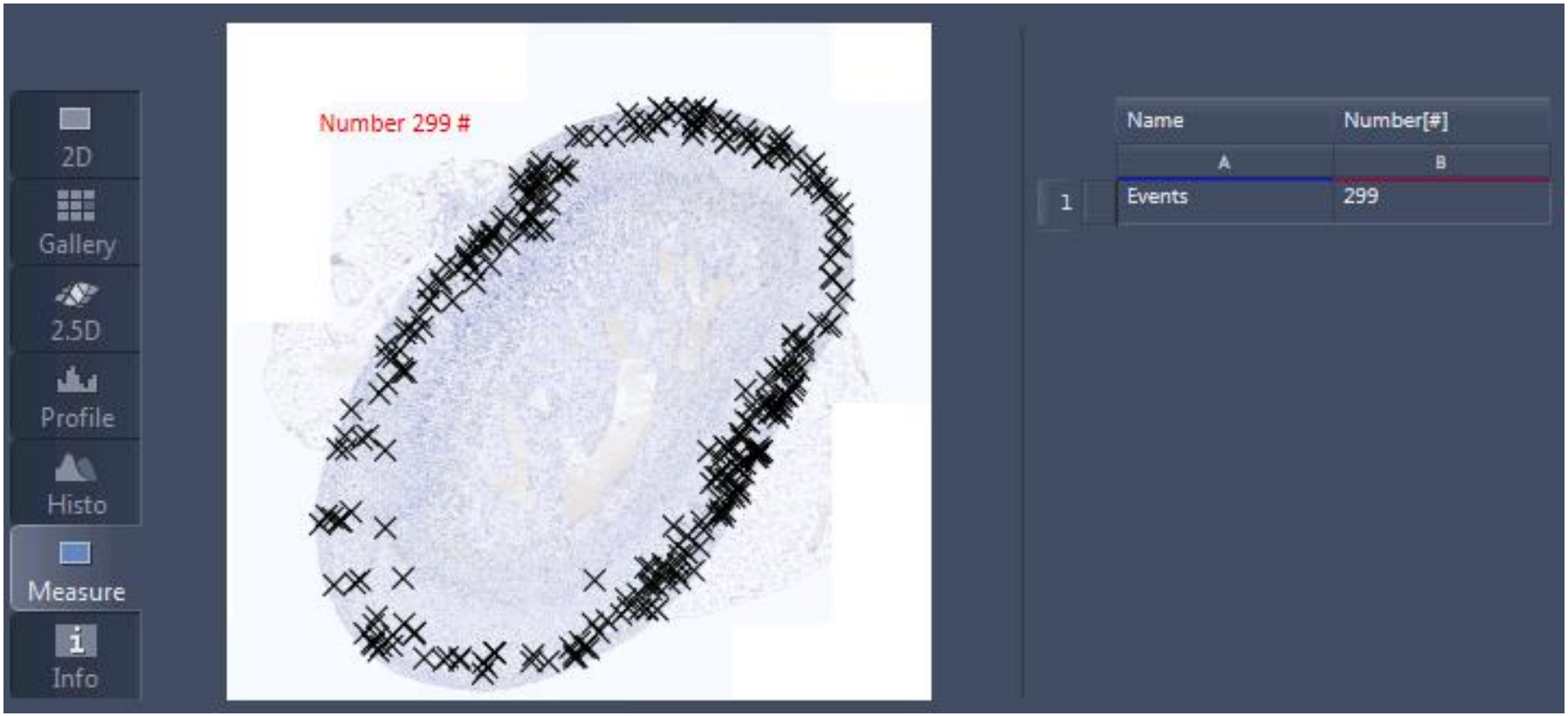


Figure 2-8. Screen grab of PCNA cell counts in Zen lite 2. *Whole section scan, numbers for PCNA positive cells can be seen on the right under name and number*

2.7 qRT-PCR

2.7.1 RNA extractions

RNA extraction was achieved through the use of an RNAeasy® mini kit (Qiagen, Crawley, UK) as per manufacturer's standard protocol for animal tissue. Whole adrenals were defrosted on ice to maintain RNA integrity. Whole adrenals were then lysed using an RLT buffer, essential for tissue lysis, and homogenised using Qiagen TissueLyser homogeniser (Qiagen, Crawley, UK) for three minutes to successfully dissociate the tissue. To allow appropriate RNA binding conditions, 70% EtOH was added to the lysate following homogenisation. Samples were then added to an RNeasy® mini spin column. The membrane present in these columns binds the RNA and allows for decontamination via a series of washes with RW1 buffer that efficiently removes biomolecules such as carbohydrates, proteins, and fatty acids. Although recommended in the protocol as an optional step, a DNAse digest was performed. This was to remove any DNA contamination that may remain in the sample. This is particularly important when running qRT-PCR. RNA was finally eluted in 30µl of sterile water, this step was performed twice to achieve maximum yield.

2.7.2 RNA quantification

A Nanodrop ND-1000 spectrophotometer (LabTech International, UK) was used to quantify the amount of RNA in the sample. Additionally to confirm the integrity of the RNA, the sample was also assessed using the 260/280 absorbance ratio. A non-contaminated sample should fall within a ratio of 2.0-2.2. Any reading out with indicates a sample contaminated with genomic-DNA.

2.7.3 cDNA Synthesis

SuperScript VILO cDNA synthesis kit (Invitrogen, Paisley, UK) was used to reverse transcribe RNA samples to create a single strand complementary DNA (cDNA) via the manufacturer's standard protocol. Each sample consisted of 10x superscript enzyme mix (2µl) responsible for the reverse transcription of single RNA strands into cDNA, 5xVilo reaction mix (4µl) which includes random primers, $MgCl_2$, and dNTPs in a buffer formulation that has been optimized for qRT-PCR, 2µg of RNA (varied amount) topped up by sterile water to 14µl. This gave a final sample volume of 20µl.

Samples were then placed in thermocycler under the following conditions: 25 °C for 10 minutes for primer extension, 42 °C for 60 minutes for cDNA synthesis, and 85 °C for 5 minutes to terminate the reaction. Only cDNA in immediate use was stored at -20 °C, whereas stock cDNA was frozen at -80 °C for further analysis.

2.8 Quantitative gene expression

To be able to quantify gene expression in experimental samples, quantitative real-time polymerase chain reaction (qRT-PCR) was achieved via the probe-based qRT-PCR method. This method provided greater sensitivity over conventional qPCR.

2.8.1 UPL probe and primer design

The qRT-PCR method used in this study requires specific probes purchased from the Universal Probe Library (UPL) (Roche, West Sussex, UK). The Universal ProbeLibrary uses the hydrolysis probe format for real-time PCR detection. The probe-based real-time PCR assay specificity is achieved through a combination of the PCR primers that specify the exact target amplicon among billions of nucleotides, and the probe. The real-time PCR probe is able to enhance specificity, and ensures that fluorescence is only generated when the target of interest is amplified. UPL probes have been designed to be no longer that 26-35 nucleotides in length, this ensures thermal stability that is essential during PCR. UPL probes are fluorogenic and permit relative quantification of a cDNA template through fluorescence detection. For genes of interest, the specific mouse probe was appropriately chosen. This probe was labelled with fluorescein (FAM) at the 5’ end and a dark quencher dye at the 3’ end which has a wavelength of 488nm. Prior to binding, the quencher dye is used to suppress the signal from the fluorophore. The qRT-PCR reaction involves annealing of the probe to the appropriate region of target DNA providing it is present in the sample being analysed, this binding occurs between the forward and reverse primers. The polymerase enzyme begins by binding to the 5’ region of the primer until it reaches the probe, which then becomes cleaved. Upon cleavage the fluorescent FAM dye is released producing a signal. This reaction occurs at the end of an amplification cycle and is what provides quantitative measurements for gene expression in a sample.

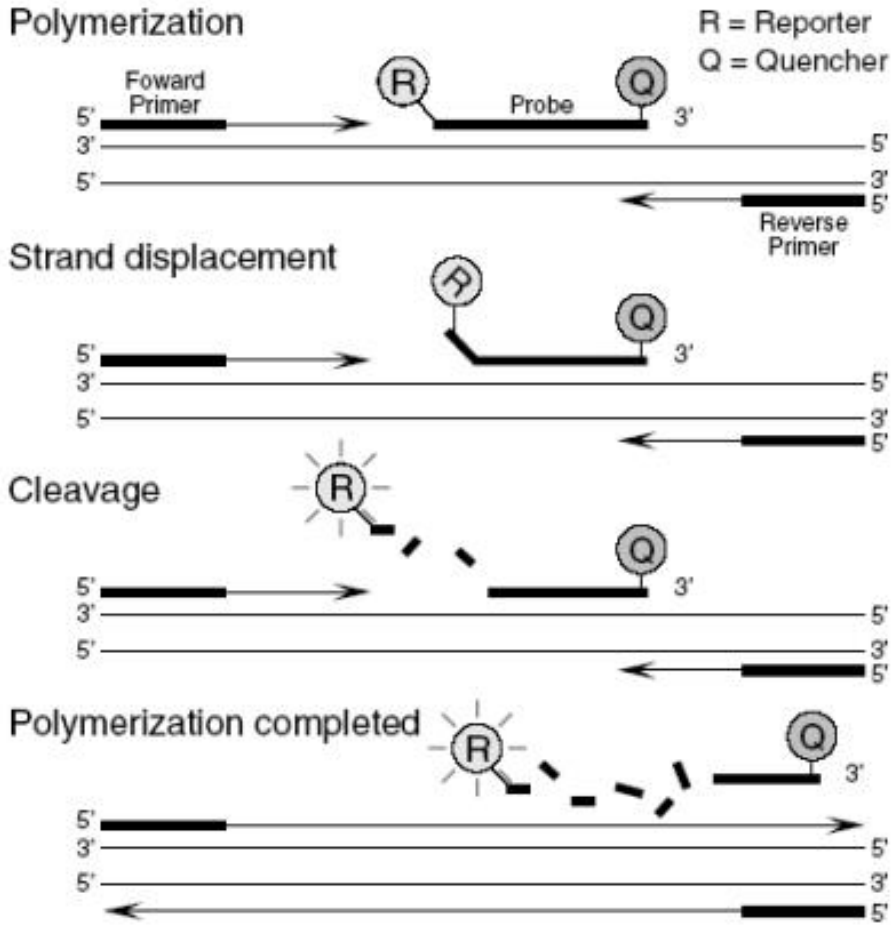


Figure 2-9. RT PCR using fluorescent reporters. *The polymerization-associated 5´–3´ nuclease activity of the DNA polymerase detaches the reporter from the quencher during the PCR reaction*

Roche provides an online service in which you can design primers which can be found at www.universalprobelibrary.com that designs and provides primer sequences and the most appropriate UPL probe for that gene of interest. These probes are also ranked in order to highlight the best probe for your set criteria. Primer pairs were then ordered from Eurofins, UK and validated prior to any use on experimental samples (2.8.4), this was to ensure appropriate cycling efficiency of the primers. The reactions carried out in this thesis was achieved through singleplex and beta-actin used as a housekeeping gene.

Transcript	5' Primer	3' primer	Roche UPL probe
Nr3c4	ttatgaagcagggatgactctg	gctgccagcattggagtt	12
Nr3c1	ccactgcaggagtctcacaa	gcaaagcatagcaggtttcc	91
Cyp21a1	ccaacctggatgagatggtt	ggattcttcccaggttccag	107
Srd5a1	gggaaactggatacaaaataccc	ccacgagctccccaaaata	41
Hsd3b1	gaactgcaggaggtcagagc	gcactgggcatccagaat	12
Akr171c8	tggccctagccaagagttt	gccaattggaaatcaaagacc	91
Akr1b7	ccaccttcgtggaactcag	cttggcctggggaagact	104
Ctnnb1	gcagcagcagtttgtgga	tgtggagagctccagtacacc	25
Gli1	ctgactgtgcccgagagtg	cgctgctgcaagaggact	84
Dax1	cgtgctctttaacccagacc	ccggatgtgctcagtaagg	3
Sf1	tccagtacggcaaggaaga	ccactgtgctcagctccac	18
Star	ttgggcatactcaacaacca	acttcgtccccgttctcc	11
Prkar1a	gctgaagtttacactgaggagga	cagccattgtcttataatcttttgg	16
Gata6	aaagcttgctccggtaacag	atcactgatgcccctaccc	21
Cyp11a1	aagtatggccccatttacagg	tggggtccacgatgtaaact	67
Hsd11b1	ggagcccatgtggtattgac	ttcaaggcagcgagacacta	69
Cyp11b1	ctggaaagtgtccatggtagc	ccccaaaaagaacaaagtgg	47
Cyp11b2	cctggagatgcatcaggtct	tccggcaacatcacagatac	98
C-kit	agcgctgcctttccttatg	cgcagatctccttggttttg	68

Table 2-3: qPCR assays used, with sequences of primers and UPL probe numbers for each assay

2.8.2 Preparing the qRT-PCR assay

To ensure reproducibility and accuracy of results all samples were plate in triplicate, all samples were additionally run with appropriate experimental controls and gene controls. To ensure there was no contamination of samples or fluorogenic compounds, plates were also run with a 'no template control' and a 'no reverse transcriptase control'. All plates when possible where run on the same 384-well plate ABI 7900, to try and account for any machine differences.

Reagent	Concentration required	Volume per triplicate reaction (384 well)	Volume per triplicate reaction (96 well)
2X Universal AB1 master mix	1x	17.5 µl	255 µl
Target gene Forward Primer	200nM	0.875 µl	1.25 µl
Target gene Reverse Primer	200nM	0.875 µl	1.25 µl
Target gene Probe	100nM	087.5 µl	1.25 µl
PCR-clean water		11.375 µl	16.25 µl
cDNA		3.5 µl	5 µl

Table 2-4 Reagents used for qRT-PCR

2.8.3 qRT-PCR analysis: ΔΔCt method

Once running of the plate was complete, the results were displayed as amplification curves which reflected the release of fluorescent product released during the amplification of a target gene, which is directly proportionate to the amount of that gene in a tissue of interest. This gene expression is shown as an amplification curve, this curve is split into three regions. The baseline region is where any background fluorescence is detected. Depending on the amount of a gene in a sample, after a certain PCR cycle, gene expression enters the exponential phase. During this phase there is an exponential increase in fluorescence detected, followed by the final phase in which the reaction plateaus. In order for a reaction to increase into the exponential phase, the fluorescence needs to be greater than the background and this increase is shown when the curve must cross the threshold. The Ct (threshold cycle) is the cycle number at

which this occurs. Lower the Ct value denotes higher level of the target gene expression in the sample. These areas are described in figure 2-10.

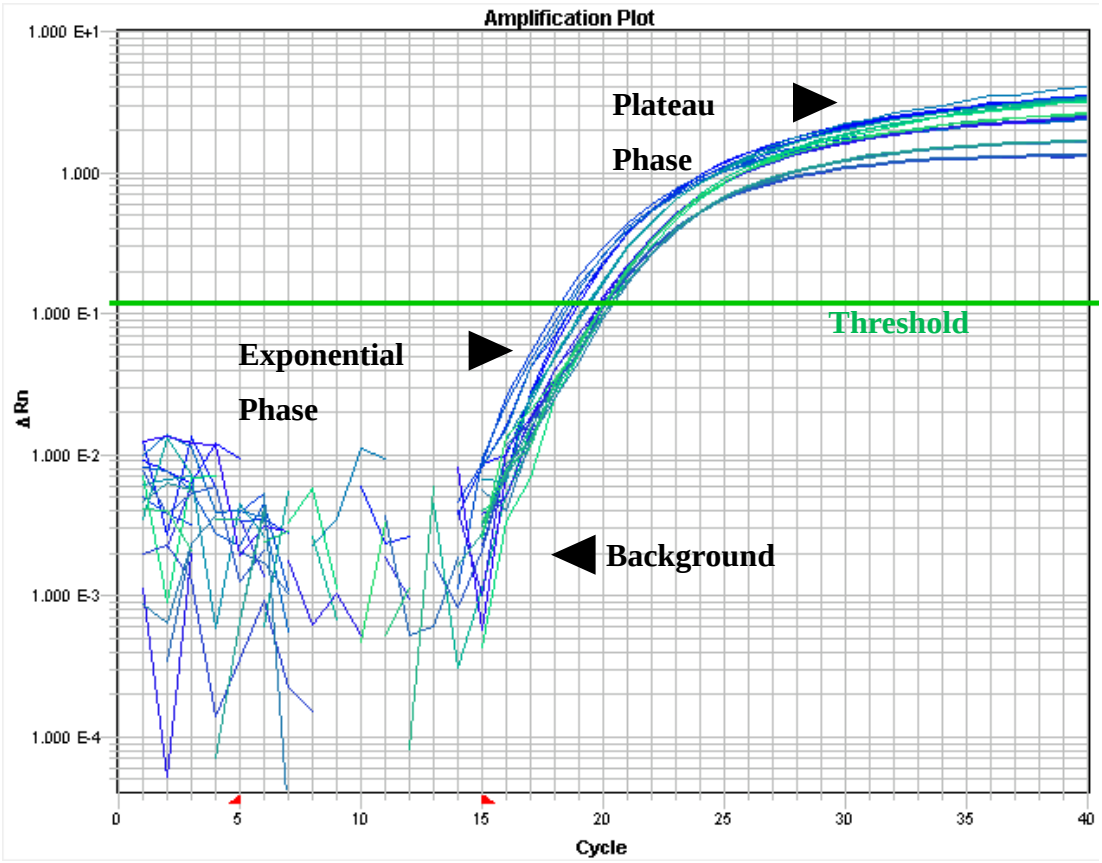


Figure 2-10. SDS file expression curve

Following this, the results need to be analysed and this is done through the comparative ΔΔCt method (265). Fold change is normalised to a reference sample such as an untreated control and relative to an internal positive control. The Ct values of experimental cohorts were first standardised to the external control to account for variation between the samples. For each sample, the ΔCt was first calculated by subtracting the the Ct for the internal positive control gene, in this case beta-actin (which is labelled with a VIC dye with a wavelength of 538) from the ΔCt of the target gene. The triplicates of each sample were then averaged and compared to a reference sample. To calculate this, the ΔCt was subtracted from the ΔCt value if the reference sample and is then referred to ΔΔCt. By using the formula $2^{-\Delta\Delta Ct}$ then provided the relative fold change of the gene of interest in a particular sample compared to a control

sample. Once the fold change was calculated the subsequent values could then be statistically analysed.

2.8.4 Primer and probe validation

Before use on experimental samples, primers had to be optimised to ensure they were cycling within an appropriate range. Appropriate amplification should be a doubling of an amplicon every cycle during the exponential phase, if this did not happen the ΔΔCt method is not valid. To test this, a five-fold dilution series was performed with wildtype adrenal cDNA (figure 2-11). Diluted cDNA was added to mastermix and plated in triplicate. The plate was run on an ABI 7900 (2.8.2) machine and the ΔCt for each concentration was calculated (1.8.3) and values were then plotted on a scatter graph with the log of the template concentrations on the X-axis. A trend line was then added and the slope of the line examined. Cycling efficiency should fall between 3 and 3.6 and anything without this range is deemed not working.

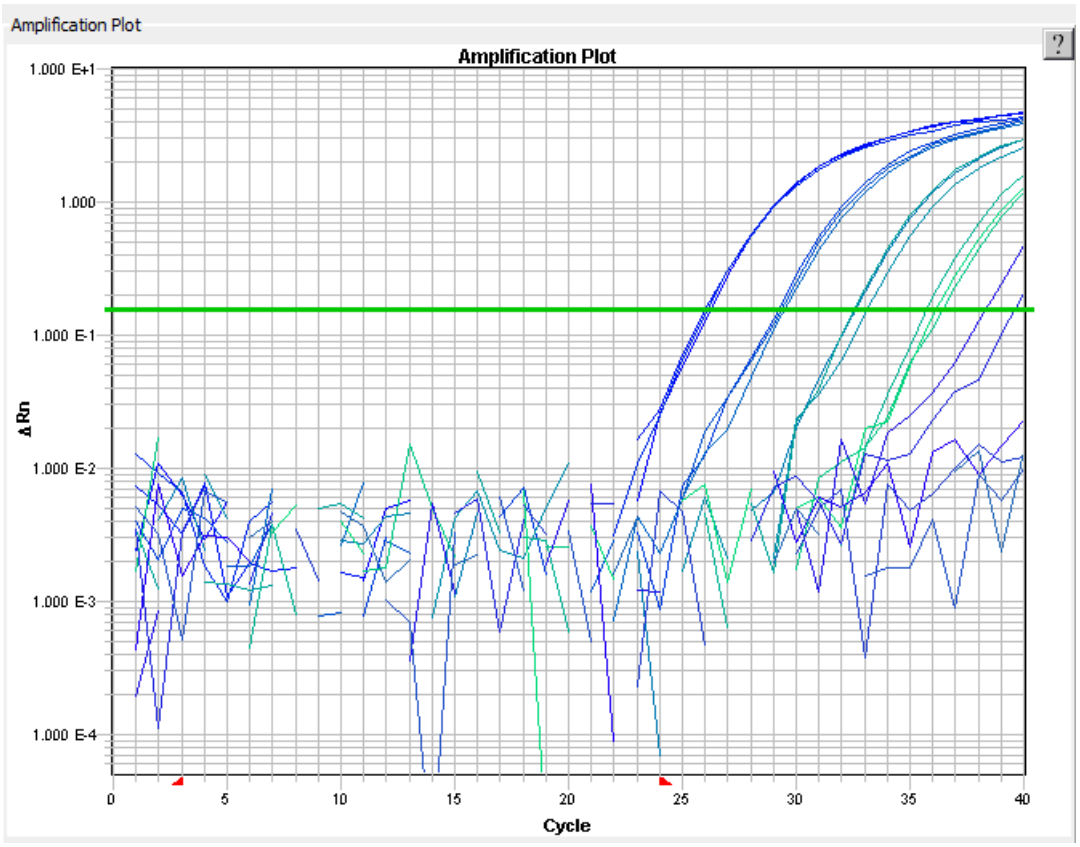


Figure 2-11. Amplification plot of series diluted primers

2.9 PCR Genotyping

To determine if an animal is carrying Cre recombinase, AR^{flox}, GR^{flox} or R26R-YFP in offspring from transgenic matings, DNA was prepared from ear clips or tail tips at weaning. From this tissue genomic DNA was extracted through a DNA-digest with 25μL TE-Tween (section 2.12.3.3) and 2μL proteinase K (10mg/ml) per ear clip. Once DNA was extracted samples were run on a thermocycler programme set to 55°C for 1 hour, then 95°C for 7 minutes followed by 25°C for 10secs on a GenePro Thermal Cycler (Hang Bioer Technology Ltd.). Tissue was then vortexed and centrifuged at 7,717g for 5 minutes. The resulting extracted DNA was carefully removed and diluted 1:10 with distilled water and stored at -20 for long-term storage.

2.9.2 PCR assay

Along with detection of Cre recombinase in samples, an internal control Interleukin-2 (IL-2) was used. This was to ensure that DNA amplification has occurred and is especially important in controlling for samples that are Cre-. The assay is set up as follows: 1μL of DNA was added to 0.1μL of each primer (Table 1), 5μL BioMix Red (Bioline, UK), and water to make up a 10ul reaction per sample. The programme used to amplify specific primer products was set up as follows: 94 °C for 3 minutes, 94 °C for 30 seconds, followed by 35 cycles at 57 °C for 30 seconds and 72 °C for 30 seconds, followed by a final incubation at 72 °C for 5 minutes. In addition to samples of interest, Cre recombinase negative DNA and dH_2O were also run on the same programme, this was to ensure there was no contamination introduced during sample preparation. This programme was used to detect the presence of Cre in the Cyp11a1-Cre line. Expected product sizes for this Cre was 100bp and 324bp for IL-2. Primer sequences listed in table 2-5.

2.9.2.1 Inheritance of floxed AR

First, a PCR mix was made as previously described and PCR was performed on Ar exon 2 on a programme for 94 °C for 3 minutes, 94 °C for 30 seconds, followed by 40 cycles of 61°C for 1 minute and 72 °C for 1 minute, followed by a final incubation at 72 °C for 5 minutes. Product size for floxed Ar was expected at approximately 852bp. Primer sequences in table 2-5.

2.9.2.2 Inheritance of floxed GR

First, a PCR mix was made as previously described and PCR was performed on Gr exon 3 on a programme for 95 °C for 5 minutes, 95 °C for 30 seconds, followed by 35 cycles at 62 °C for 1 minute, 72 °C for 1 minute, 72 °C for 5 minutes, 72 °C for 5 minutes). Expected product sizes were 240bp for wild type GR and 300bp for recombined GR. Primer sequences listed in table 2-5.

2.9.2.3 YFP insertion in R26R-EYFP mice

To identify if Rosa26 mice were carrying an insertion of YFP, a PCR mastermix was set up as described in 2.9.2. Both Rosa26 wildtype and YFP insertion regions were amplified on a PCR programme as follows: 94 °C for 3 minutes, 94 °C for 30 seconds, followed by 36 cycles at 58 °C for 1 minute, 72 °C for 1 minute, 72 °C for 2 minutes, 72 °C for 5 minutes and finally 25 °C for 10 seconds). Expected product sizes were 600bp for wild type Rosa26 and 320bp for YFP insertion. Primer sequences are listed in table 2-5.

Transgene	**Primer sequence**
Cre forward	GCGGTCTGGCAGTAAAAACTATC
Cre reverse	GTGAAACAGCATTGCTGTCACTT
Interleukin-2 forward	CTAGGCCACAGAATTGAAAGATCT
Interleukin-2 reverse	GTAGGTGGAAATTCTAGCATCATCC
AR floxed forward	GCTGATCATAGGCCTCTCTC
AR floxed reverse	TGCCCTGAAAGCAGTCCTCT
GR floxed forward	GGCATGCACATTACTGGCCTTCT
GR floxed reverse 1	GTGTAGCAG CCAGCTTACAGGA
GR floxed reverse 2	CCTTCTCATTCCATGTCAGCATGT
Rosa26 wild type	GGAGCGGGAGAAATGGATATG
Rosa26 wild type	AAGTCGCTCTGAGTTGTTAT
YFP insertion	AAGACCGCGAAGAGTTTGTC

Table 2-5. Primer sequences.

2.10 Statistical analysis

All statistical analysis was carried out on GraphPad Prism version 7 (GraphPad Software Inc, San Diego, CA, USA). All values are presented with error bars as standard error of the mean ±SEM. Data was first analysed via D'Agostino-Pearson normality test to determine if the data had a normal distribution, if this was the case an appropriate parametric test was performed based in the samples size, number of groups being compared and if post-hoc analysis was required . Parametric analysis used in the thesis involved a two-tailed, unpaired t-test was used to compare two groups, one-way ANOVA to compare multiple groups and two-way ANOVA to compare multiple groups and treatments. If a post-hoc analysis was required, a Tukey's analysis was performed that allowed comparison of the mean of each group to the means of every other group or a Dunnett's test if comparing multiple groups to a single control group. If groups failed the normality test, a Mann-Whitney U test was performed. For multiple group analysis The Kruskal–Wallis test was carried out for 3 or more groups in case of non-parametric data.

2.11 PET/CT Scanning

Computed tomography (CT scans are a useful tool in examining bone structure and density. From the images constructed from CT scans measurements can be taken that can reveal changes in shape, structure and density.

2.11.1 Ex-vivo analysis of spinal curvature

Interrogation spinal curvature was achieved via CT scanning. Whole body micro CT images of mice was acquired. 8 groups of animals were be included in this study with 5 animals per group between male and female controls and knockout mice. Collected CT images were reconstructed and analysed using PMOD software. Mice were carefully positioned head first prone in the scanner bed for collection of coronal and sagittal plane radiographs using a nanoPET/CT scanner (Mediso, Hungary) and the following settings: side or top view, X-ray energy of 50 kVp, exposure time of 300 ms and maximum field of view. Coronal and sagittal plane radiographs were used for animal positioning for high-resolution microCT imaging, which was acquired using

the following parameters: semi-circular full trajectory, 720 projections, maximum zoom, tube voltage of 50 kVp, exposure time of 300 ms, binning of 1:4. Nucline software (Mediso, Hungary) was used to reconstruct microCT images using the following parameters: voxel size medium, slice thickness medium and cosine filter with 100% cutoff (combined voxel resolution: isotropic 251 μm). The Nucline software was also used to assess gross anatomical measurements and to measure the magnitude of the largest scoliotic and thoraco-lumbar kyphotic curves, according to the Cobb method (266)/Fergunson method (267).

2.12 Commonly used solutions

2.12.1 Immunohistochemistry

2.12.1.1 Harris's haematoxylin

25 ml of 10% haematoxylin in absolute alcohol was added to 500 ml of 10% warm aluminum potassium sulphate solution. The solution was heated to boiling point and 1.25 g of mercuric oxide (1.25g) was added slowly. The haematoxylin solution was plunged into ice to cool before filtering it into a staining dish. Finally, glacial acetic acid was added (4 ml per 100 ml haematoxylin).

2.12.1.2 Eosin

An aqueous eosin solution was made by dissolving 1% w/v eosin in water, and an alcohol solution was made by dissolving 1% w/v eosin in methylated spirit. The solutions were mixed at 1 part alcohol to 3 parts aqueous and filtered. 0.5 ml/L of formaldehyde was added to prevent bacterial growth.

2.12.1.3 Acid alcohol

1% concentrated hydrochloric acid was added to 70% ethanol.

2.12.1.4 Scotts tap water

0.2% w/v potassium hydrogen carbonate and 2% w/v magnesium sulphate were added to tap water

2.12.1.5 Bouin's fixative

Bouin's fixative is a well-established formaldehyde based fixative, which appears bright yellow in colour (purchased from Triangle Biomedical Sciences Ltd, Lancashire, UK).

2.12.1.6 Normal sera

Normal chicken and goat sera were obtained from Biosera, UK. Normal serum: phosphate-buffered saline: bovine serum albumin (NS: PBS: BSA) NS from the animal of choice was diluted 1 in 4 with PBS (section 2.9.2.1) containing 5% BSA. Normal serum: tris-buffered saline: bovine serum albumin (NS: TBS: BSA) NS from the animal of choice was diluted 1 in 4 with TBS (section 2.9.2.2) containing 5% BSA.

2.12.2 Buffers

2.12.2.1 Tris-Buffered Saline

For 1L of 10X TBS stock (0.2M Tris, 1.5M NaCl, pH 7.6), 24g Tris HCl (Sigma Aldrich, Dorset, UK), 5.6g Tris base (Sigma Aldrich, Dorset, UK) and 88g NaCl (Sigma Aldrich, Dorset, UK) were dissolved in 900mL deionised water and adjusted to pH 7.6 using concentrated HCl or NaOH to decrease or increase pH respectively. The volume was then made up to 1L and the solution stored at 4oC TBS solution was very carefully adjusted to pH 7.4 using concentrated HCl and stored as a stock solution at 4°C.

2.12.2.2 Citrate Buffer

For 2L of Citrate buffer, Citric acid monohydrate (Sigma, Dorset, UK) 42.02g and 1900mL dH_2O was combined. The pH of citrate buffer was adjusted to pH6.0 or pH9.0 using saturated NaOH solution and used at 0.01M by dilution (1:10) in dH_2O.

2.12.3 DNA extraction

2.12.3.1 Tris-acetate-EDTA (TAE)

To make a 50x solution, 242 g of Tris base, 57.1 mL glacial acetic acid (BDH, UK) and 100 mL 0.5M EDTA were made up to 1 L with ultra-filtered water. The solution was adjusted to pH 8.5 by a saturated solution of NaOH and the stock solution was diluted to 1x with ultra-filtered water before use.

2.12.3.2 0.5M EDTA

186.1 g EDTA was added to 800 ml of ultra-filtered water. The solution was adjusted to pH 8.0 with a saturated solution of NaOH to allow the EDTA to dissolve then diluted to 1 L with ultra-filtered water.

2.12.3.3 Tris-EDTA-Tween (TE-Tween)

Fresh Tris-EDTA-Tween was prepared before use. This was achieved by adding 2.5mL 1M Tris (2.9.1.1), 100µL 0.5M EDTA and 250uL tween 20 (Sigma, UK) to 48mL deionised water. After thorough mixing, the solution was sterile filtered through a 0.22µm syringe filter unit (Merk Millipore, USA).

Chapter 3: Androgens and androgen receptor can work together and independently to regulate aspects of adrenal function and morphology

3.1 Introduction

Androgens have been well documented as important regulators of male health, primarily in the maintenance and development of male sexual characteristics (268). However, a decline in circulating androgens has also been associated with co-morbidities such as obesity (269) cardiac disease (270) and metabolic syndrome (271). Androgens primarily exert their effects through their cognate androgen receptor (AR), a nuclear receptor that binds to testosterone and dihydrotestosterone and translocates into the nucleus to regulate gene transcription (272). Androgens and the androgen receptor can also act independently of each other to affect cellular processes (273). 90% of androgen production in men occurs in the testis with the remainder produced in the zona reticularis (ZR) of the adrenal cortex (274).

Previous research has focussed upon the body-wide impact of adrenal androgens, however whilst AR is abundantly expressed in the adrenal cortex of both rodents (275), (276) and humans (120), surprisingly little is known about androgen action on the adrenal cortex itself. This gap in our understanding is at least in part due to the perceived lack of suitable animal models. Mice have largely been overlooked as a model system as their adrenals are unable to produce androgens due to lack of 17α hydroxylase and 17, 20 lyse activity and therefore do not have a zona reticularis (10), (111). However, historical studies using castrated mice showed that removal of androgens leads to the redevelopment of an additional cortex zone (23). The foetal adrenal is thought to provide the necessary stem/progenitor cells to populate the adult adrenal cortex in human and mice (277) (20). These foetal cells are maintained for a period postnatally and regress differently depending on species and sex. In the human this zone is known as the 'foetal zone', and the mouse homologue termed the 'X-zone' (57), (278). The mechanisms underpinning the regression of the X-zone and its purpose and maintenance postnatally still aren't clearly understood

Chapter 3: Androgens and androgen receptor can work together and independently to regulate aspects of adrenal function and morphology

The redevelopment of the X-zone following castration and the abundant expression of AR in the mouse adrenal suggests that androgens are important regulators of the adrenal cortex and that the mouse, despite not producing adrenal androgens, has utility for dissecting the role of androgen signalling within the adrenal. Previous global knockouts have ablated AR from the whole body and then examined the adrenal (162), however the caveats of this, like many global knockout models that focus on the endocrine system, is that the adrenals both produce and respond to multiple endocrine stimuli that may also be disrupted in a global knockout model. Being able to attribute any phenotype to perturbed endocrine signalling locally, versus from disruption of the wider hypothalamic-pituitary-axis is extremely difficult.

For these reasons an adrenal specific androgen receptor knockout model (Ad-ARKO) was generated that completely ablates AR from the adrenal cortex in foetal and adult life (253), allowing investigation of adrenal AR signalling. Due to the ability of androgen receptor and androgens to act independently of each other, additional models of castrated wild-type, castrated AR ablated, and hCG treated animals were investigated to fully elucidate androgen and AR action in the adrenal. Results demonstrate that not only are androgens signalling via androgen receptor required for X-zone regression, but androgens and androgen receptor also work independently to regulate multiple aspects of cortex function and morphology.

Chapter 3: Androgens and androgen receptor can work together and independently to regulate aspects of adrenal function and morphology

3.1.1 Aims

- To establish androgen receptor localisation in the human and mouse adrenal cortex
- To create adrenal specific androgen receptor knockout mice through the use of a *Cyp11a1*-Cre mouse line bred to AR floxed mice.
- To define the impacts on adrenal development following AR ablation
- To determine the impacts on cortex morphology, function and stress response following AR ablation in adulthood

3.2 Results

3.2.1 Androgen receptor is widely expressed throughout the human and mouse adrenal cortex

Previous studies have examined the expression of AR in the adrenal, however this was restricted to transcript, protein localisation was not established (279). To establish where AR is localised in the adrenal cortex, human and mouse adrenals were examined following immunohistochemistry. AR protein localisation is observed in human foetal adrenals (Fig. 3-1A) and human adult adrenals (Fig. 3-1B). Similar analysis of the mouse foetal adrenal shows AR localisation at (e)13.5 (Fig. 3-2A). AR protein is expressed in the postnatal mouse adrenal when examined at d12, d21, d35 and d80 (Fig. 3-2B). AR is localised at the cortex medulla boundary at d12, by d21 AR is localised throughout the zona fasciculata (ZF) and from d35 onward AR can be seen in the ZF and zona glomerulosa (ZG). These data confirm the expression of AR in similar locations and timings in both human and mouse adrenals during development and in adulthood, suggesting that despite not producing androgens, the mouse adrenal has human relevance as a model system for study of the within-adrenal response to androgen stimulation.

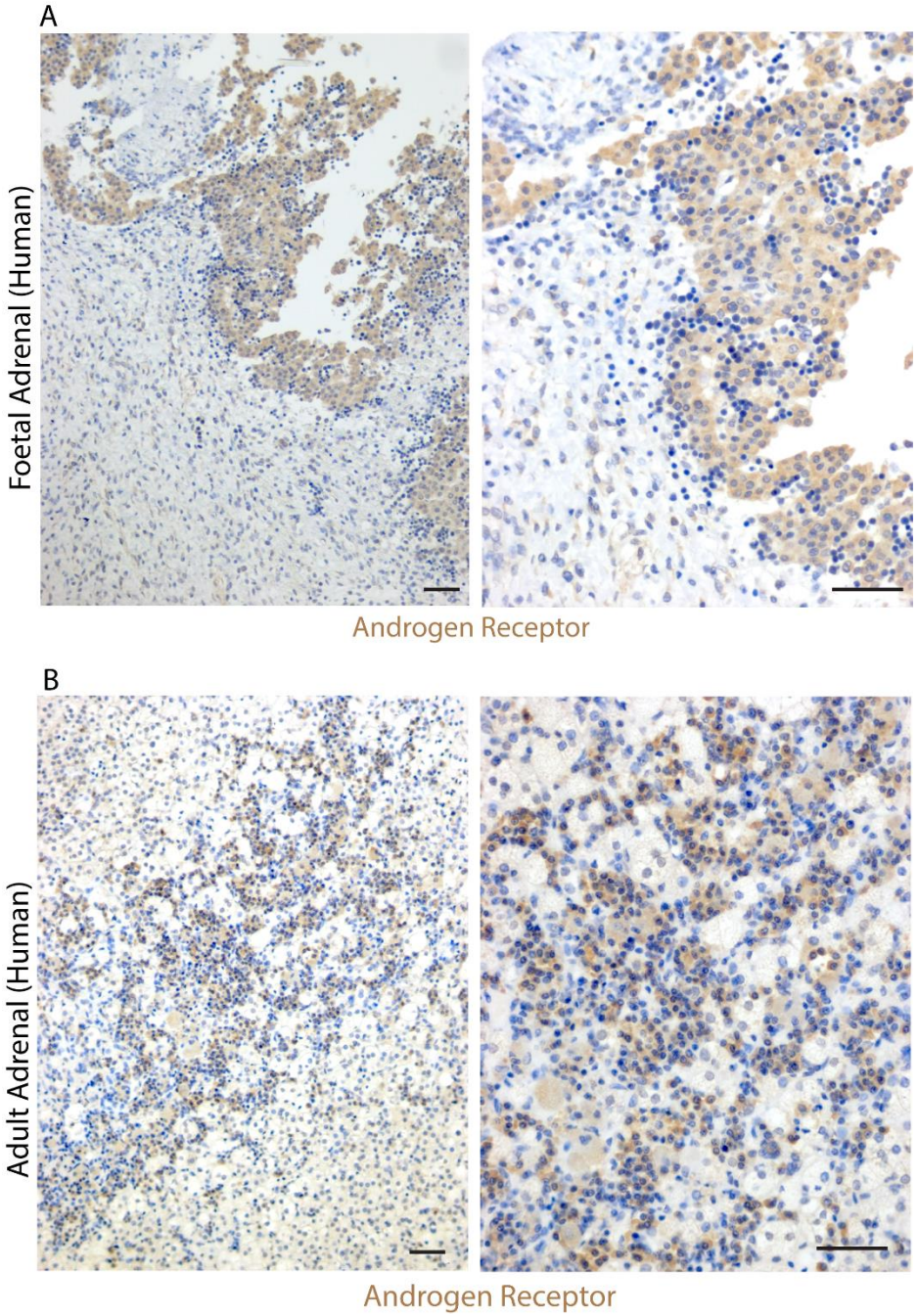


Figure 3-1. Androgen receptor expression in the human adrenal cortex. ***(A)*** *Androgen receptor immunostaining details AR expression in human foetal adrenals. Staining can be seen in the region of the definitive zone. N=2* ***(B)*** *Androgen receptor immunostaining details AR expression in adult human adrenals. Staining can be seen in the zona reticularis region. N=2. Scale Bars 50µm.*

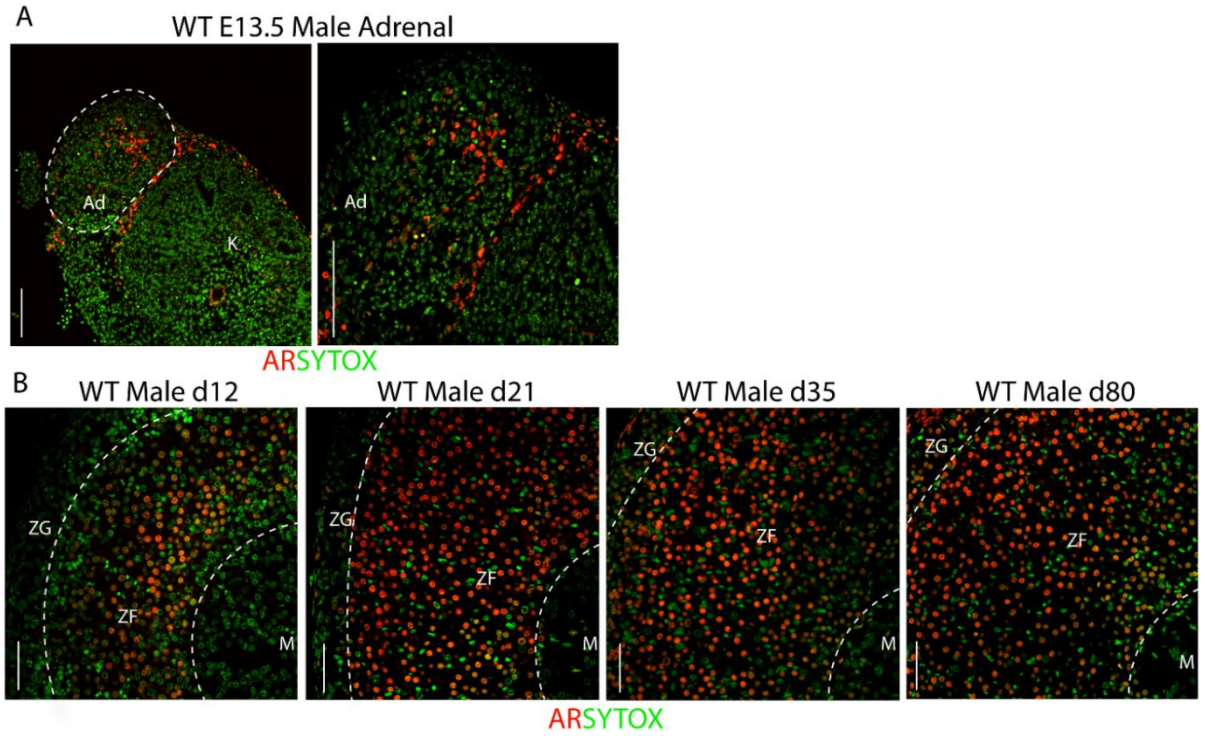


Figure 3-2. Androgen receptor expression in the mouse adrenal cortex. (A) *Androgen receptor localisation can be detected at e13.5 in embryonic adrenals, with staining in the central region of the foetal adrenal. 'Ad' denotes foetal adrenal, boundaries denoted by white dotted lines.* ***(B)*** *Expression observed during development shows a migration from the inner most cortex/medulla boundary (WT d12) and migrates outwards with age (WT d21, d35, d80). By d80 androgen receptor expression can be observed across the whole cortex. N=5. Red: androgen receptor protein, Green: sytox counterstain. Scale bar 50µm. Annotations; M=medulla, ZF= zona fasciculata, ZG = zona glomerulosa.*

3.2.2 Confirmation of ablation of androgen receptor from the mouse adrenal

The Smith lab has previously described a novel method to ablate target genes from steroidogenic cell types through the development of a GFP-Cre-GC targeted to the mouse *Cyp11a1* locus to drive Cre Recombinase expression (253). The lab previously established that this Cre line targets less than 20% of testicular Leydig cells (253), and that loss of AR from Leydig cells has no discernible impact on circulating testosterone levels. The paper also highlights no discernible impact from off target effects in unspecified tissues. Immunostaining shows that Ad-ARKO mice have complete ablation of AR protein throughout the adrenal cortex (Fig. 3-3A), demonstrating that this model permits the cell-specific dissection of androgen signalling in the adrenal cortex. Interestingly, the adrenal capsule does not express *Cyp11a1* therefore *Cyp11a1*-Cre does not target these cells, resulting in AR expression still being maintained in the capsule (253, 44), an important positive control for downstream studies.

3.2.3 Ad-ARKO mice have enlarged adrenals and failed X-zone regression

Ad-ARKO adrenal weights are similar to controls in d12 or d21 mice, however, at d35 and d80 there is a significant increase in adrenal weight in the Ad-ARKO compared to controls (Fig 3-3B). Examination of gross adrenal morphology reveals the presence of a thicker and larger X-zone in d12 and d21 male Ad-ARKO adrenals compared to controls. From d21 to d35 involution and regression of the X-zone occurs in male mice, leaving only two cortical zones in adulthood (280). However, in Ad-ARKO mice the X-zone fails to regress during this period and remains present in adulthood, occupying a large proportion of the adrenal cortex (Fig. 3-4A). Female mice also have an X-zone that remains present until first pregnancy in adulthood (34). As Ad-ARKO male adrenals have an X-zone, they were compared to age-matched WT female mice, to determine whether loss of AR had ‘feminised’ the male adrenal. However, the X-zone in Ad-ARKO males appear much larger and occupies a greater portion of the cortex compared to WT females, suggesting loss of AR in males does not simply feminise the male adrenal.

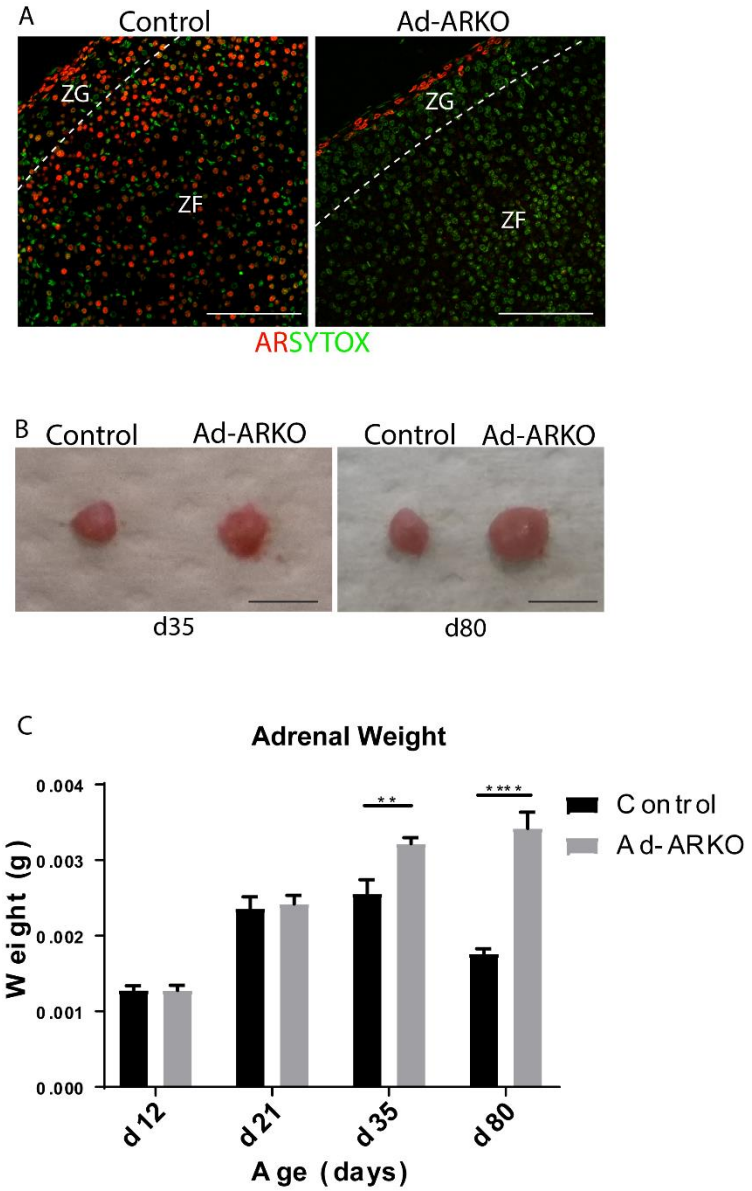


Figure 3-3. Ad-ARKO mice show an increased adrenal weight and size. ***(A)*** *Immunofluorescence staining confirms ablation of AR from adrenal cortex in d80 mice (Scale Bars 50µm). N=5 Red: androgen receptor protein, Green: sytox counterstain.* ***(B)*** *Observations of whole tissue show Ad-ARKO males have larger adrenals at d35 and d80 (Scale bar 1mm). N=5* ***(C)*** *Ad-ARKO males have significantly larger adrenals from d35 (Two-Way ANOVA; ** p<0.001, n=5, Tukeys post-hoc analysis, error bars SEM), this difference in size is maintained into adulthood (Two-Way ANOVA; **** p<0.0001, n=5, Tukeys post-hoc analysis, error bars SEM). Annotations; M=medulla, ZF= zona fasciculata, ZG = zona glomerulosa. N=8.*

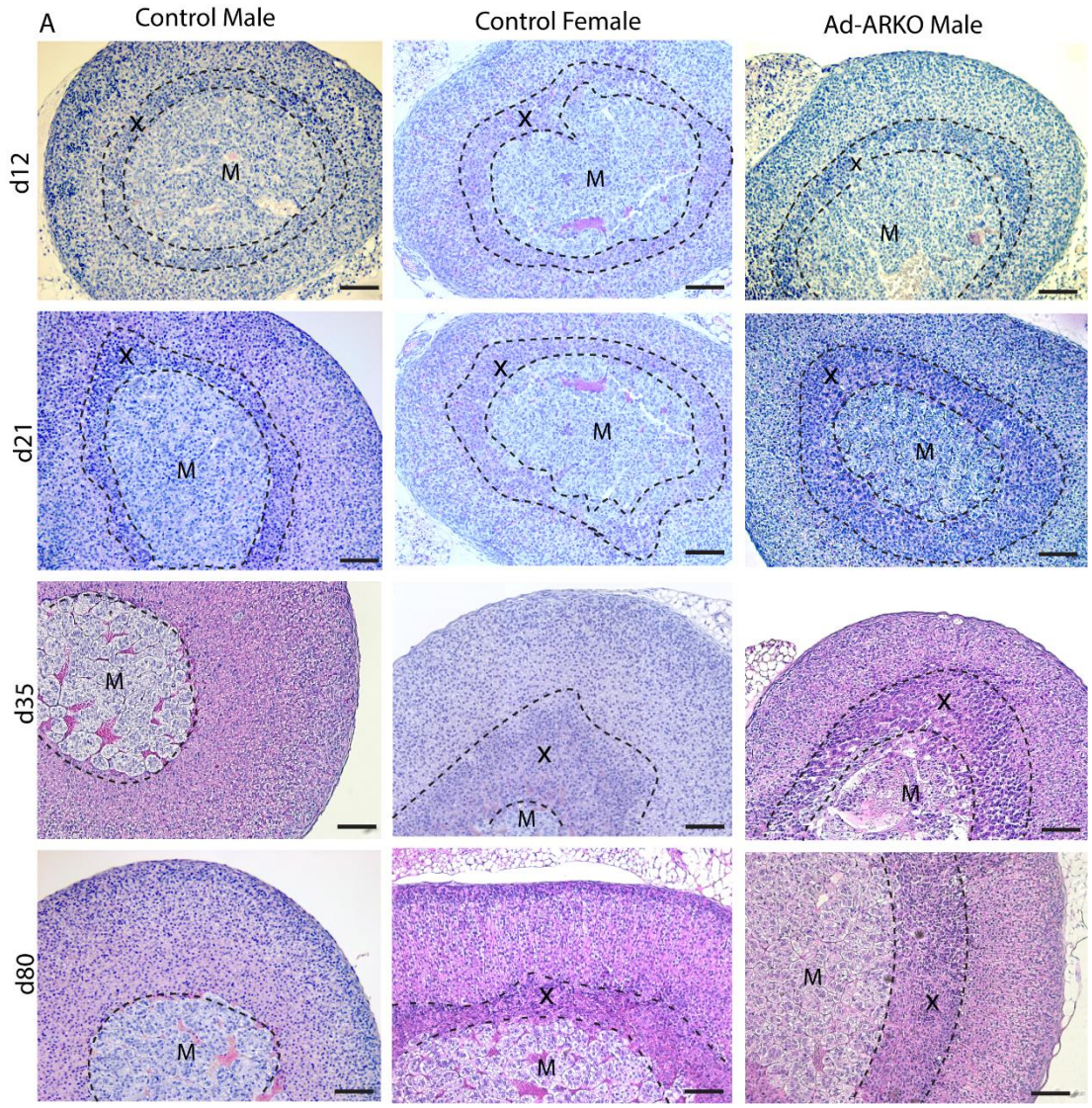


Figure 3-4. The X-zone of Ad-ARKO mice persists into adulthood. (A) *Adrenal histology in d12 and d21 mice do not appear to show any major differences compared to controls. Full regression of the X-zone is usually complete by d35 which is demonstrated in controls, however there is an additional inner cortical zone still present in Ad-ARKO males at this time point. This inner cortical zone can again be observed in d80 males, with this extra cortical zone occupying a large proportion of the adrenal cortex. Scale Bars 50µm. Annotations; X= X-zone, M= Medulla. N=5.*

3.2.4 Androgens are essential for normal morphology of the male adrenal

To frame the parameters of the study, examination of the gross morphology of the adrenal in models of high (hCG stimulation) and low (castration) circulating testosterone were performed. Treatments with hCG have been shown to result in elevated circulating testosterone following a single dose (acute), with daily treatments able to maintain high levels of testosterone for two weeks (chronic), beyond this time point hCG specific antibodies are generated rendering the treatment ineffective and circulating testosterone returns to normal (158). Disruption of androgen signalling leads to regeneration of the foetal X-zone region, consistent with previous studies (23), however, acute or chronic increased circulating androgens did not impact the adult adrenal cortex or X-zone morphology, implying that an upper limit to androgen stimulation exists (Fig. 3-5 A, B). For these reasons, the remainder of the chapter focuses on the impact of loss of androgens and its impact on the adrenal cortex.

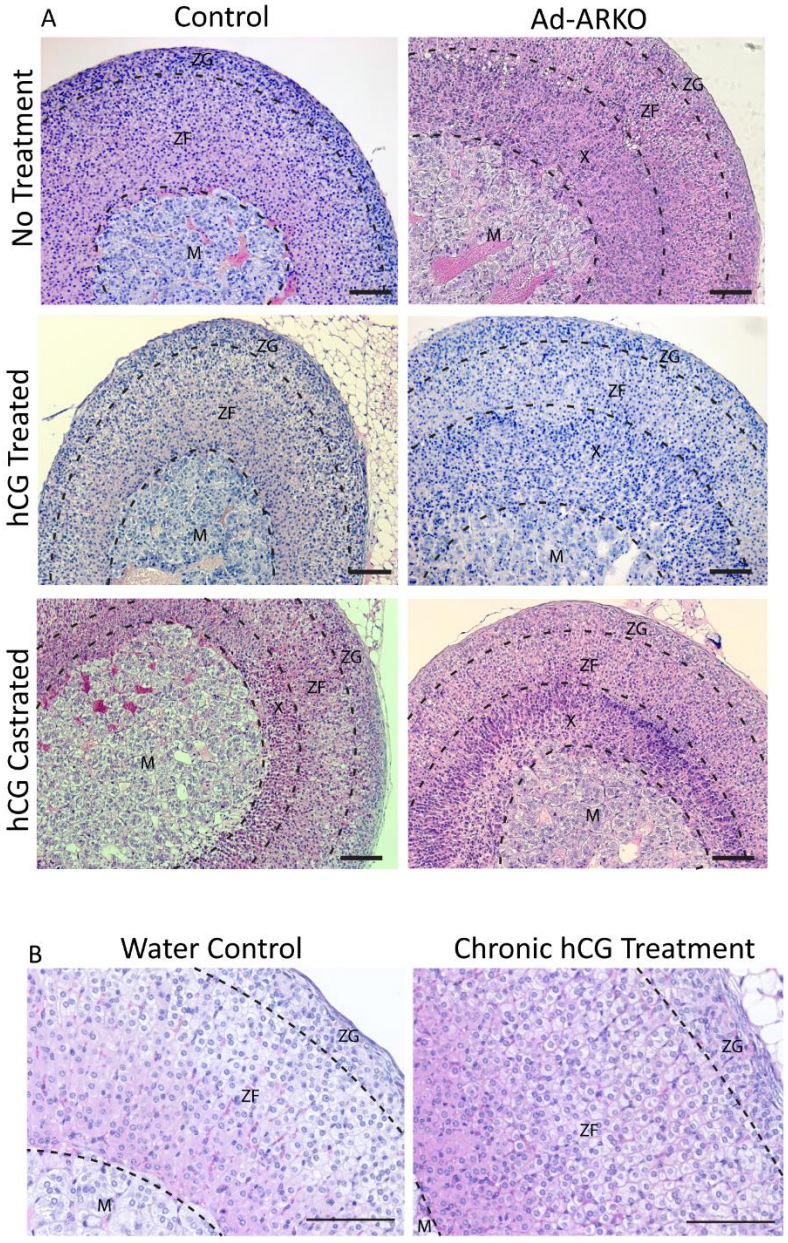


Figure 3-5. Increased circulating androgens does not impact adrenal morphology. (A) *Morphology analysis of d80 control and Ad-ARKO treated with a single dose of hCG and control castrated and Ad-ARKO castrated treated with hCG show no further changes to morphology in the cortex or X-zones compared to non-treated controls. N=5* ***(B)*** *Chronic treatment with hCG in d80 WT males showing no impact on the morphology of the adrenal cortex. Scale bars 50µm. Annotations; M=medulla, ZF= zona fasciculata, ZG = zona glomerulosa, X= X-zone. N=5.*

3.2.5 20 alpha-HSD expression confirms maintenance of foetal cells in adulthood in Ad-ARKO

Androgens can act independently of their receptor to modulate transcriptional activity (144, 273), therefore additional cohorts were included that consisted of Bl6 castrated and Ad-ARKO castrated cohorts in the study (both cohorts) castrated at d80. To determine receptor and ligand-independent effects on adrenal regulation. These additional cohorts also have a significant increase in adrenal weight compared to controls (Fig. 3-6A). Investigation of the histology of Bl6 castrated, and Ad-ARKO castrated mice revealed the presence of X-zones in their cortex (Fig. 3-6B). Immunohistochemistry for 20-α-hydroxysteroid dehydrogenase (20 alpha-HSD), a specific marker for the foetal cells that make up the rodent X-zone (40, 281), confirms that the additional cortical zone present in the experimental cohorts is composed of foetal cells (Fig. 3-6 C, D). Although all experimental cohorts have developed X-zones there are clear differences in 20 alpha-HSD localisation (Fig. 3-7A). This suggests that X-zone cells and the cortex are impacted differently depending on whether circulating androgens or AR have been targeted. In addition to this, as females maintain an X-zone into adulthood, a control female adrenal was included in the comparison (Fig. 3-7B). Morphology of the female adrenal again is different from what is observed in the male cohorts, with differences in size and X-zone cell morphology. For these reasons detailed cortex measurements were taken.

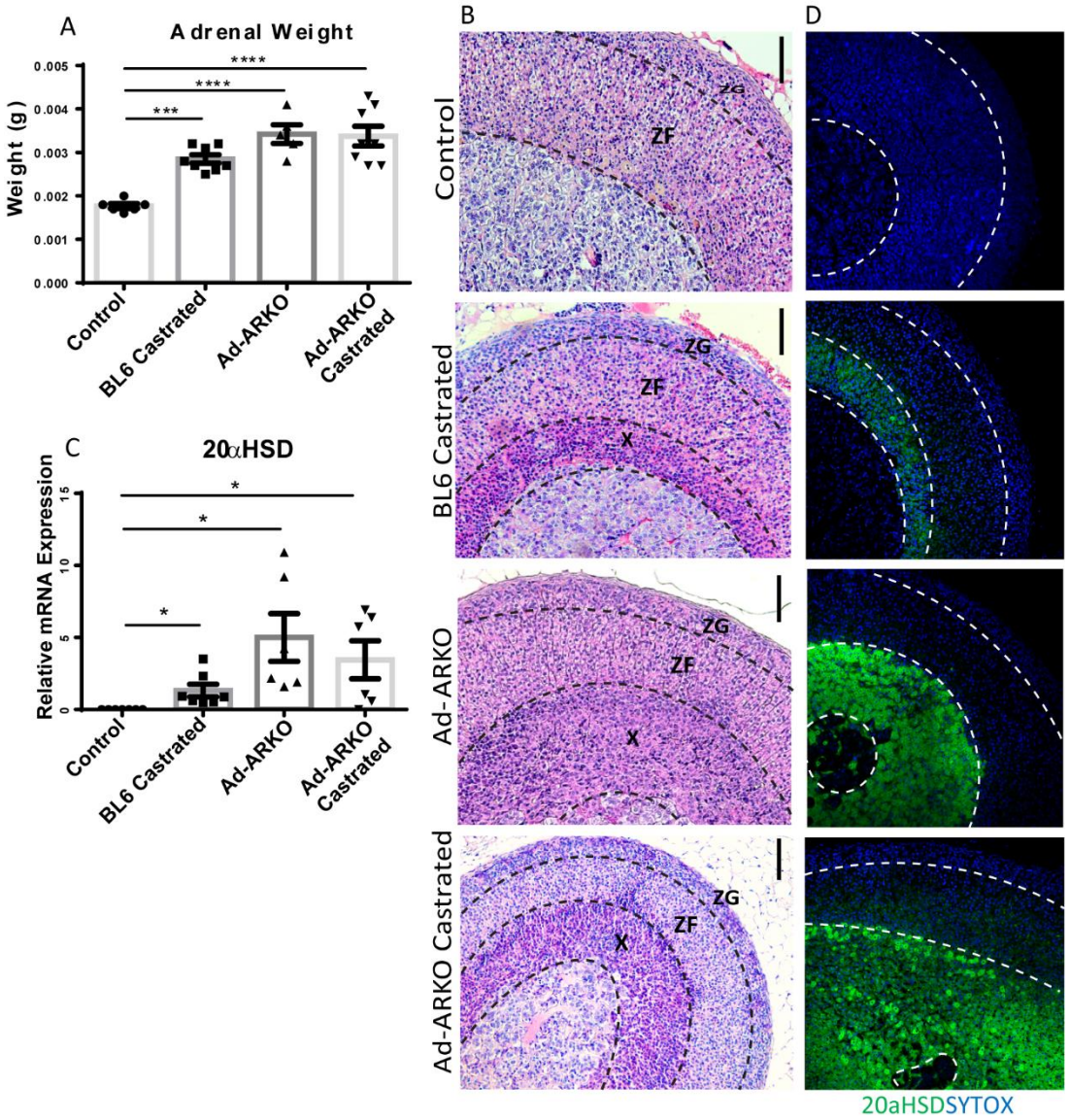


Figure 3-6. X-Zone markers confirm the maintenance of foetal cells in Ad-ARKO Mice. ***(A)*** *In all experimental cohorts an increase in weight was detected when compared to controls (one-way ANOVA; n=8, *** p<0.0001, **** p<0.0001, **** p<0.0001, Tukeys post-hoc analysis, error bars SEM* ***(B)*** *All experimental cohorts have developed and additional cortical zone. N=5* ***(C)*** *qRT-PCR data confirms 20 alpha HSD expression in castrated, Ad-ARKO and Ad-ARKO castrated mice (one-way ANOVA; n=7-8, *p<0.05, *p<0.05, *p<0.05, Tukeys post-hoc analysis, error bars SEM).* ***(D)*** *Immunostaining for 20alpha-HSD (green) confirms that the extra cortical zone developed in all experimental cohorts. N=5. Red: 20 alpha HSD protein, Green: sytox counterstain. Scale Bars 50µm. Annotations; M=medulla, ZF= zona fasciculata, ZG = zona glomerulosa, X= X-zone.*

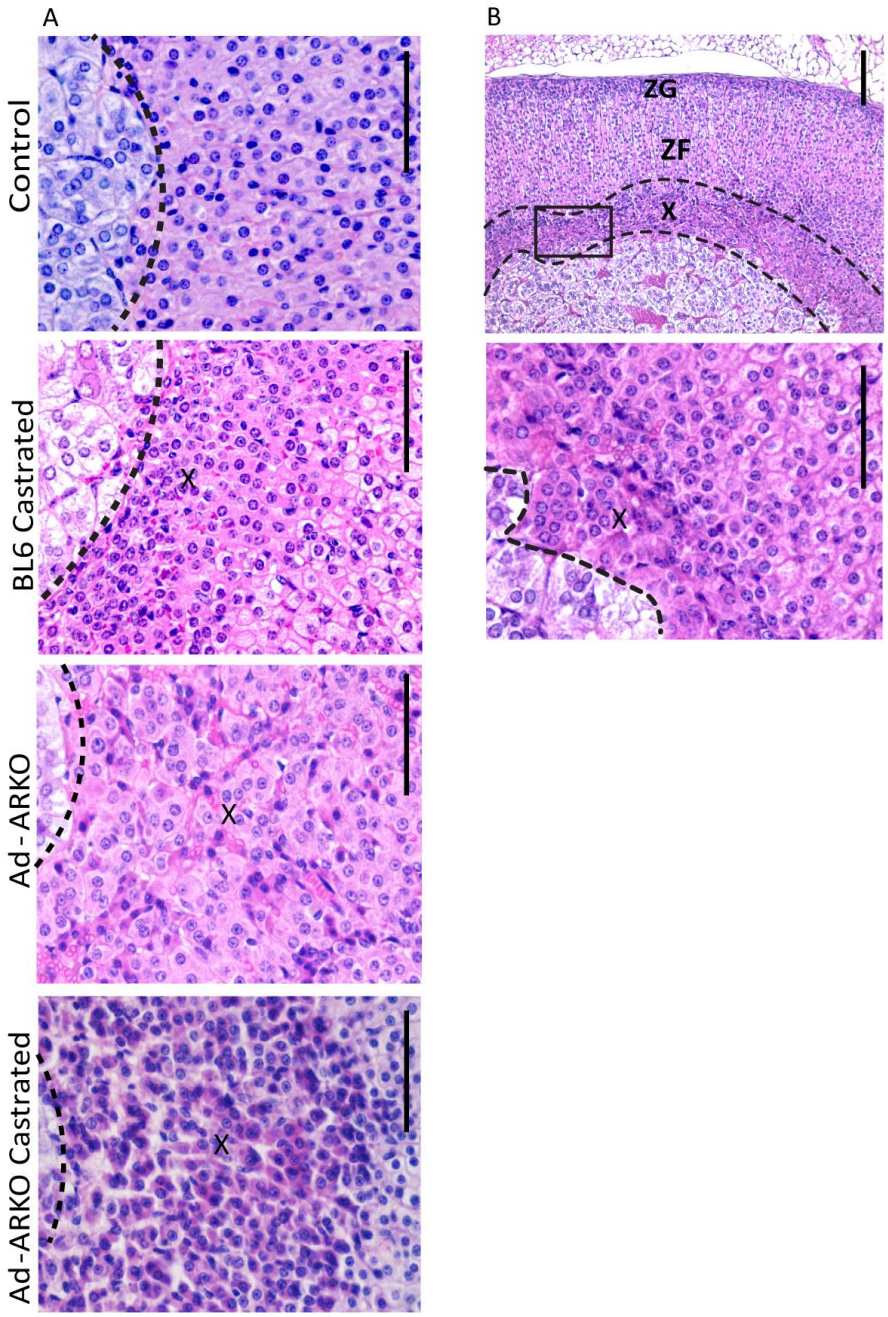


Figure 3-7. Closer inspection of X-zone reveals differences in morphology. (A) *Further investigation of X-zones present in these cohorts show morphological differences in X-zone size and cell density. Cohorts with removal of circulating androgens have shrunken cytoplasm's compared to AR ablation alone. They also appear to occupy different proportions of the cortex. N=5* ***(B)*** *Control female adrenal highlighting X-zone cells for comparison. Scale Bars 50µm. Annotations; ZF= zona fasciculata, ZG = zona glomerulosa, X= X-zone. N=5.*

3.2.6 Morphologies of X-zones in Bl6 castrated, Ad-ARKO and Ad-ARKO castrated show distinct characteristics

To distinguish differences in morphology, the X-zone depth, cell density and cortex depth were measured. X-zones in Ad-ARKO and Ad-ARKO castrated mice are significantly larger than Bl6 castrated mice (Fig. 3-8A). X-zones in Bl6 castrated and Ad-ARKO castrated have significantly denser X-zones than Ad-ARKO mice (Fig. 3-8B). Combined measurements of the zona glomerulosa (ZG) and zona fasciculata depth (ZF) show that Ad-ARKO mice have a significantly smaller ZF/ZG ratio compared to both castrated cohorts (Fig. 3-8C). Measurements of complete cortex depth show no differences, demonstrating that the X-zones are occupying different proportions of the cortex (Fig. 3-8D). These data coupled with morphological analysis suggest that AR and circulating androgens are able to act on the adrenal gland independently of each other, as well as in concert.

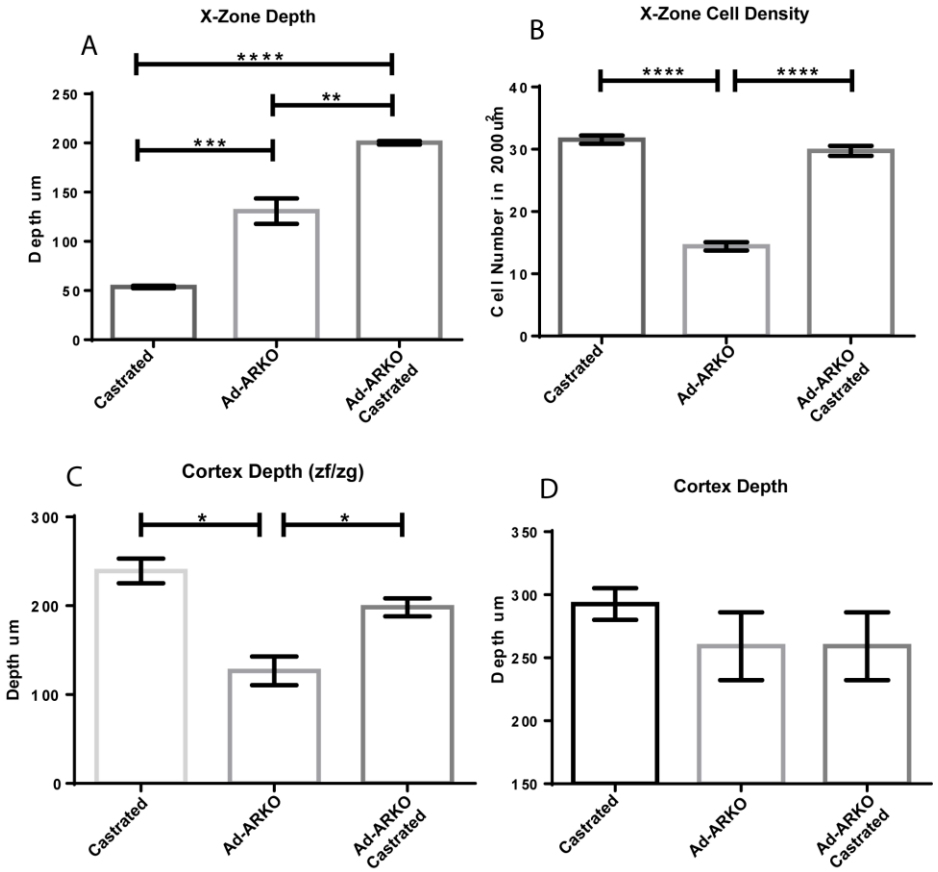


Figure 3-8. X-zones in BL6 Castrated, Ad-ARKO, and Ad-ARKO Castrated mice have distinct morphological differences. ***(A)*** *X-Zone measurements of depth showed that Ad-ARKO and Ad-ARKO Castrated mice have a significantly larger X-Zone than BL6 Castrated mice (one-way ANOVA; ** p<0.001, *p<0.05, Tukeys post-hoc analysis, error bars SEM).* ***(B)*** *The X-zone in BL6 Castrated mice is significantly denser than in Ad-ARKO mice, however upon castration of Ad-ARKO males, this cell density changed to be similar to Bl6 castrated mice, (one-way ANOVA; **** p<0.0001, ****p<0.0001, Tukeys post-hoc analysis, error bars SEM).* ***(C)*** *A significantly smaller zg/zf depth is observed in Ad-ARKO mice compared to the castrated cohorts (one-way ANOVA; * p<0.05, *p<0.05, Tukeys post-hoc analysis, error bars SEM).* ***(D)*** *There was no significant difference in the overall cortex depth between the three cohorts. X-zone measurements are presented as averages, for individual counts refer to Appendix figure 1.*

3.2.7 Disruption to AR signaling results in an increased stress response in the adrenal cortex

To establish the impact of loss of AR signalling on adrenal function key biomarkers of the adrenal stress response pathway were analysed; circulating corticosterone concentration (282), adrenal *Mc2r* expression (283) and circulating pituitary ACTH concentration (249) in Bl6 castrated, Ad-ARKO and Ad-ARKO castrated mice. Ad-ARKO mice show an increase in circulating corticosterone compared to controls, which is normalised by castration (Fig. 3-9A). Interrogation of *Mc2r* transcript shows a significant increase in all cohorts when compared to controls (Fig. 3-9B). Circulating ACTH shows no changes in any cohort examined, suggesting that the increase in corticosterone is from disruption within the adrenal and not a result of signalling from the pituitary (Fig. 3-9C). These results reveal an increased stress response in Ad-ARKO mice that is resolved upon castration, suggesting circulating androgens are acting independent of AR to stimulate the adrenal cortex.

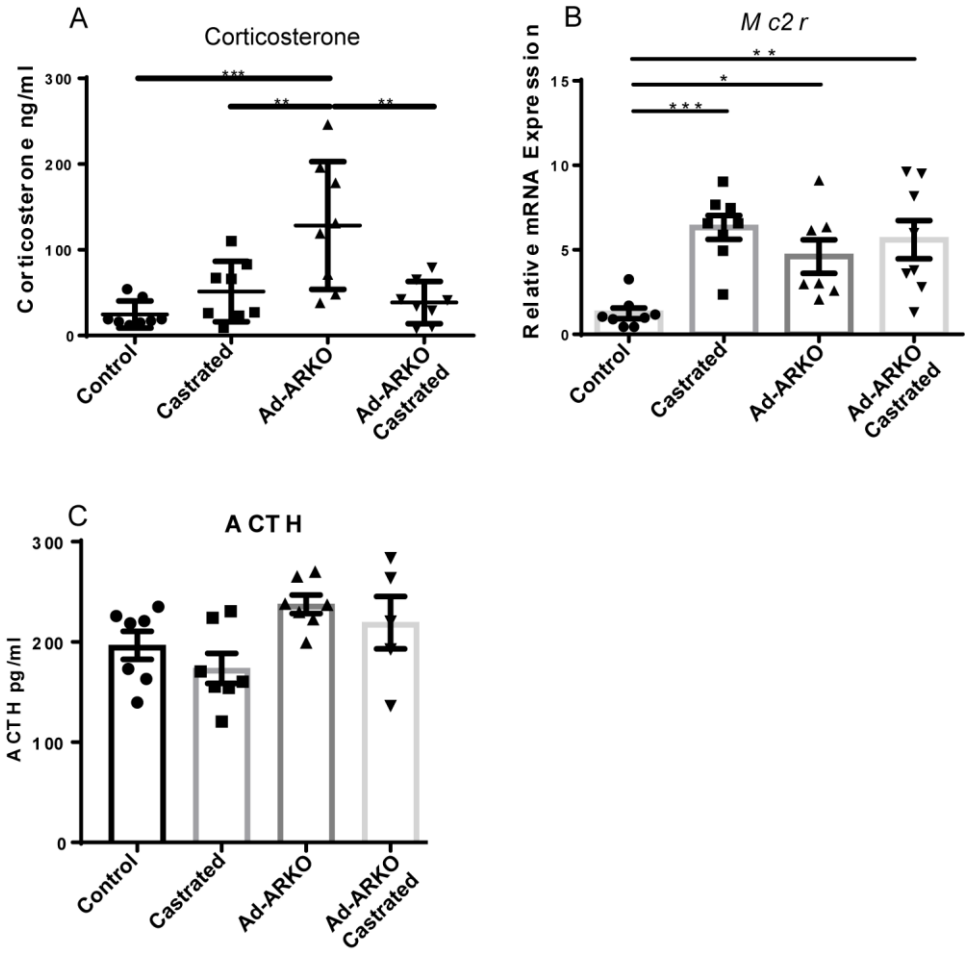


Figure 3-9. Disruption to androgen signalling results in an increased stress response in the adrenal cortex. (A) *Circulating corticosterone levels are elevated in Ad-ARKO males compared to controls (One-way ANOVA; n=8, **p<0.001, Tukeys post-hoc analysis, error bars SEM), however this is not observed in the other experimental cohorts investigated.* ***(B)*** *Mc2r gene expression shows an increase in all cohorts compared to controls (one-way ANOVA; n=8, *** p<0.0001, *p<0.05, **p<0.001, Tukeys post-hoc analysis, error bars SEM).* ***(C)*** *Circulating ACTH levels shows no changes in experimental cohorts compared to controls.*

3.2.8 Loss of AR or androgens results in changes in key developmental and cortex renewal genes

Failed X-zone regression in Ad-ARKO and/or castrated mice shows that AR signalling is required in the prepubertal adrenal for normal development and cortex maintenance in adulthood. Therefore, 20 alpha-HSD staining at key time points in the postnatal developing adrenal, and expression of *Sf1*, *Dax1 and Prkar1a*, all key genes well characterised as important drivers of the development and maintenance of the adrenal cortex (57, 65, 59) were analysed. Previous studies have suggested that any influence androgens have on the adrenal cortex does not occur until puberty (280), however, investigation of 20 alpha-HSD localisation in developing adrenals shows that the X-zone in Ad-ARKO mice is much larger than controls before, during and after puberty (Fig. 3-10A). Transcript analysis of *Sf1* shows a significant increase in all experimental cohorts when compared with controls at d80 (Fig. 3-10B). *Dax1,* a downstream target of *Sf1* (57), shows no increase in Bl6 castrated or Ad-ARKO mice, however, there is a significant increase in transcript in Ad-ARKO castrated mice (Fig. 3-10C). *Prkar1a* gene encodes for a subunit of an enzyme called protein kinase A, essential in cAMP signalling. A knockout of this gene in the adrenal cortex has been associated with the development of foetal cells (29). Due to a similar phenotype being observed in Ad-ARKO and/or castrated mice, investigation of transcript levels of *Prkar1a* in experimental cohorts was carried out and results showed a significant downregulation of this gene in all experimental cohorts when compared with controls (Fig. 3-10D). Together these data suggest an important role for androgens during postnatal adrenal development, and support the hypothesis that genes thought to be regulating the X-zone may be direct or indirect downstream targets of AR signalling.

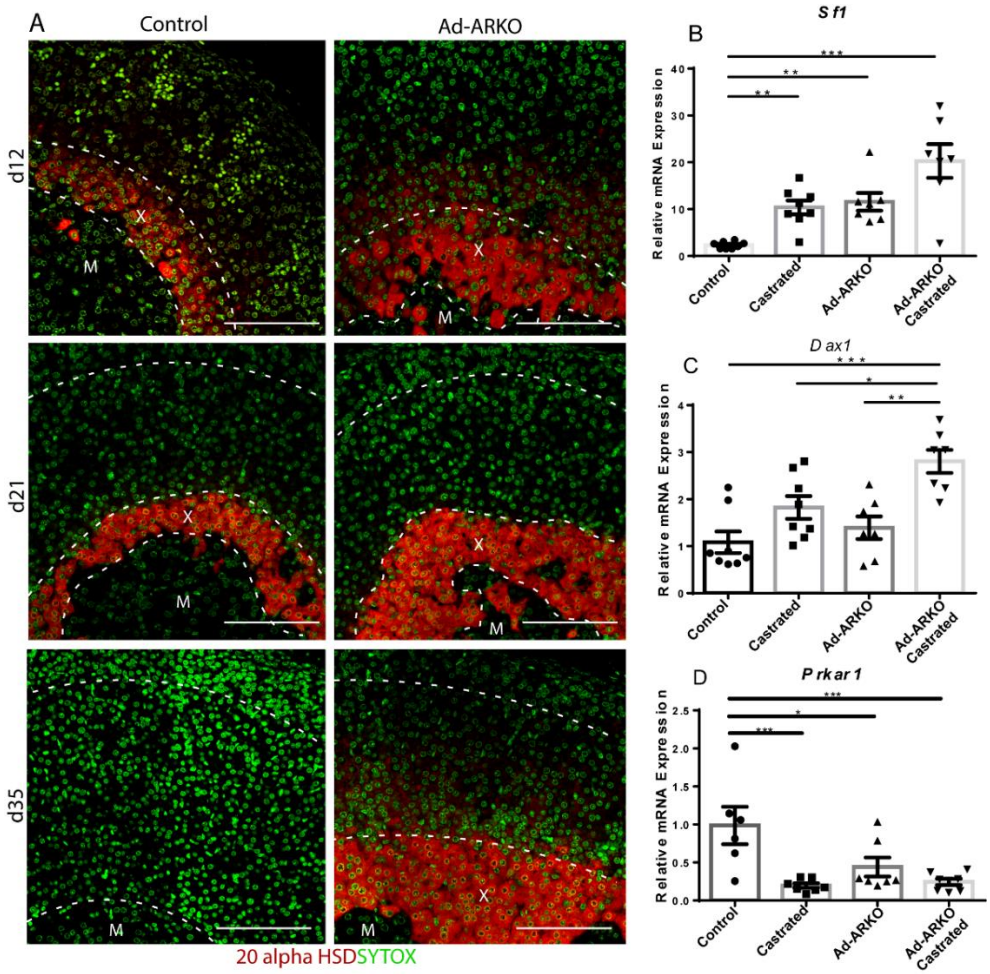


Figure 3-10. Loss of androgen signalling results in changes in key developmental and cortex renewal genes. ***(A)*** *Protein localisation of 20 alpha-HSD in the developing postnatal adrenal. It can be seen that from d12 that the X-zone in Ad-ARKO mice is more developed and larger in size compared to controls. N=5. Red: 20 alpha HSD protein, Green: sytox counterstain.* ***(B)*** *Sf1 gene expression shows an increase in all experimental cohorts. (One-way ANOVA; n=8, ** p<0.001, **p<0.001, ***p<0.0001, Tukeys post-hoc analysis, error bars SEM), however this increase was not observed in Ad-ARKO castrated males.* ***(C)*** *Dax1 gene expression has increased in Ad-ARKO castrated males, however this is not observed in the experimental cohorts (One-way ANOVA; n=8, *** p<0.0001, Tukeys post-hoc analysis, error bars SEM).* ***(D)*** *Prkar1 gene expression has decreased in all experimental cohorts compared to controls (one-way ANOVA; n=8, *** p<0.0001, *p<0.05, ***p<0.0001, Tukeys post-hoc analysis, error bars SEM). Annotations; M=medulla, X= X-zone.*

3.2.9 Disruption to androgen signalling results in changes to steroid enzyme gene expression and protein localisation.

To ascertain the impact of loss of androgens or AR signalling on adrenal steroidogenesis, analysis of the expression of a panel of key steroidogenic enzyme genes relevant to adrenal function and their protein localisation was performed. *Cyp21a1* is involved with the biosynthesis of the steroid hormones aldosterone and corticosterone and is usually produced by ZF cells (15). *Cyp21* disruption has been associated with diseases such as congenital adrenal hyperplasia (41). An increase in *Cyp21a1* expression is observed in the adrenals of Ad-ARKO and Ad-ARKO castrated mice when compared to controls (Fig. 3-11A). Interestingly, in all experimental cohorts CYP21A1 protein localisation is observed in all cortex zones compared to controls where protein is solely localised to the ZF (15) (Fig. 3-11B). This could lead to overactive steroidogenesis in the adrenal glands. *Hsd3b1* encodes for the enzyme 3β-HSD and is responsible for the biosynthesis of progesterone from pregnenolone, 17α-hydroxyprogesterone from 17α-hydroxypregnenolone, and androstenedione from dehydroepiandrosterone (DHEA) in the adrenal gland (284), and is expressed throughout the adrenal cortex. Investigation of *Hsd3b1* shows no changes in transcript levels in any experimental cohort compared to controls (Fig. 3-11C) but shows differences in protein localisation in all experimental cohorts compared to controls. Bl6 castrated mice have X-zones that express 3βHSD. Ad-ARKO mice show sporadic localisation of 3βHSD in the X-zone, however, when Ad-ARKO mice are castrated no 3βHSD localisation can be observed in the X-zone (Fig. 3-11D). ZF marker *Akr1b7*, shown to play an important role in the detoxification of lipid peroxidation by-products (40, 285) shows no change in gene expression in any of the cohorts (Fig. 3-11E). However, AKR1B7 protein is no longer observed in the adrenal cortex in Bl6 castrated and Ad-ARKO castrated mice. Interestingly, AKR1B7 is still expressed in Ad-ARKO mice (Fig.3-11F), suggesting androgens promote this expression via an AR-independent mechanism. Additional genes that were analysed as part of this study but revealed no changes are detailed in table 3-1.

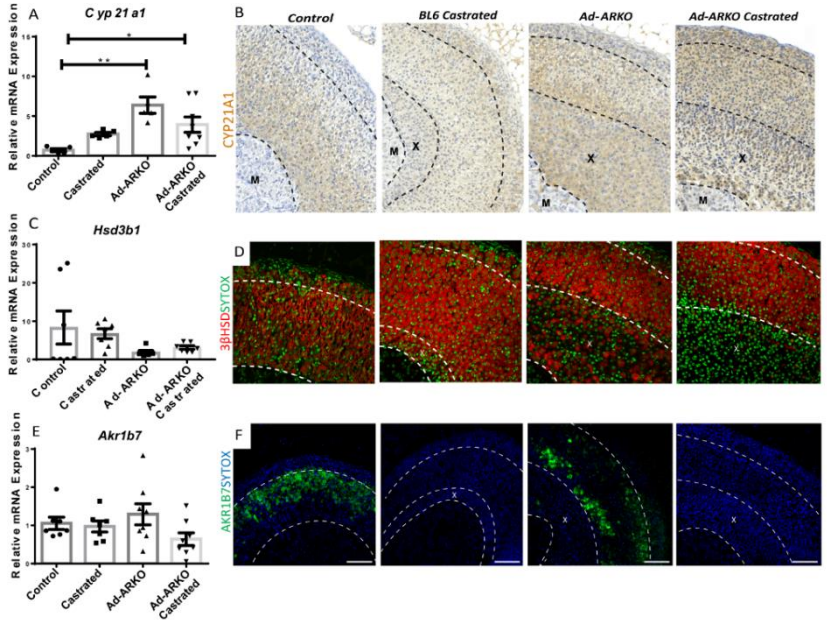


Figure 3-11. Androgens can work independent of their receptor to regulate steroid enzyme localization. ***(A)*** *Transcript analysis of Cyp21a1 shows an increase in expression in Ad-ARKO and Ad-ARKO castrated males mice (one-way ANOVA; * p<0.05, **p<0.001, Tukeys post-hoc analysis, error bars SEM).* ***(B)*** *Interrogation of CYP21A1 protein localisation shows in control adrenals that staining is localised to the ZF, however upon androgen disruption the staining can be observed in all zones in the cortex. N=5* ***(C)*** *Hsd3b1 gene expression shows no changes in transcription following androgen loss of androgen signalling in any of the experimental cohorts. (one-way ANOVA, Tukeys post-hoc analysis, error bars SEM).* ***(D)*** *3bHSD immunostaining (Protein; red, sytox counterstain; green) shows the X-zone in castrated mice still positive for 3bHSD, however expression in Ad-ARKO X-zones is more sporadic with less positive cells and Ad-ARKO castrated mice show no expression in their X-zones. N=5.* ***(E)*** *Akr1b7 gene expression shows no difference in expression any of the cohorts. (one-way ANOVA, Tukeys post-hoc analysis, error bars SEM).* ***(F)*** *AKR1b7 immunostaining (Protein; Green, sytox counterstain; blue) is lost in both cohorts that have been castrated, however AKR1B7 expression can still be observed in Ad-ARKO mice. N=5. Scale Bars 50µm. Annotations; M=medulla, X=X-zone.*

Gene	**Detected?**	***P* value**
Cyp11b1	Yes	0.402
Cyp11b2	Yes	0.8311
Star	Yes	0.8060
Prlr	Yes	0.4013
Pa2g4	Yes	0.7940
Tgfb3	Yes	0.7381
Ctnnb1	Yes	0.4445
Gata6	Yes	0.3521
Wnt4	Yes	0.6777
Gli1	Yes	0.2615
Cyp19a1	No	--
ERα	No	--
ERβ	No	--
Lhr	No	--
Fshr	No	--
Gata4	No	--
Wnt1	No	--

Table 3- 1. Table of genes analysed in this study. *Genes investigated in this study that revealed no changes in Ad-ARKO mice compared to littermate controls. Genes that showed no detection in adrenal samples had testis, ovary or pituitary controls to ensure that the qPCR had worked.*

3.2.10 Disruption to apoptosis and undifferentiated cell clusters can be observed in Ad-ARKO castrated mice.

GR has been shown to interact directly with AR in various tissues to regulate cellular processes (203, 205, 252, 286), to determine if loss of adrenal AR signalling impacts GR, transcriptional and protein analysis was performed. Investigation of GR in the adrenal cortex shows an increase in *Gr* transcript in Bl6 castrated and Ad-ARKO castrated mice (Fig. 3-12A). GR immunostaining identified clusters of GR positive cells in the outer cortex of Ad-AKRO castrated mice that extend from the capsule to the ZF (Fig.3-12B) which are not observed in Bl6 castrated or Ad-ARKO mice. Immunostaining of AR shows protein localisation in the same capsule cell clusters observed in Fig. 3-12B in Ad-ARKO castrated mice (Fig. 3-12D), this suggests that these cells have not expressed the Cre and are not steroidogenic, additionally, it is known from AR/GR double immunohistochemistry analysis of control mice that AR and GR are not expressed in the same cells, AR is located in the large steroid producing cells, whereas GR can be seen in endothelial like cells (Fig. 3-13). This suggests that these cell clusters in the outer cortex are cells that are undifferentiated (287). Due to failed regression of X-zone cells from the cortex during puberty and cell cluster development in the outer cortex of Ad-ARKO castrated mice, immunostaining of cleaved caspase 3 was used to determine if there is any disruption to apoptosis. Immunostaining of cleaved caspase 3 shows that in controls adrenal cells undergo apoptosis at the cortex-medulla boundary, but in BL6 castrated and Ad-ARKO mice no cleaved caspase positive cells can be seen. However, Ad-ARKO castrated mice show aberrant apoptosis throughout the cortex (Fig.3-12E). These results suggest that, in the absence of androgens and/or AR, the adrenal is failing to appropriately differentiate its cortex zones in addition to disruption to cell clearance from the cortex.

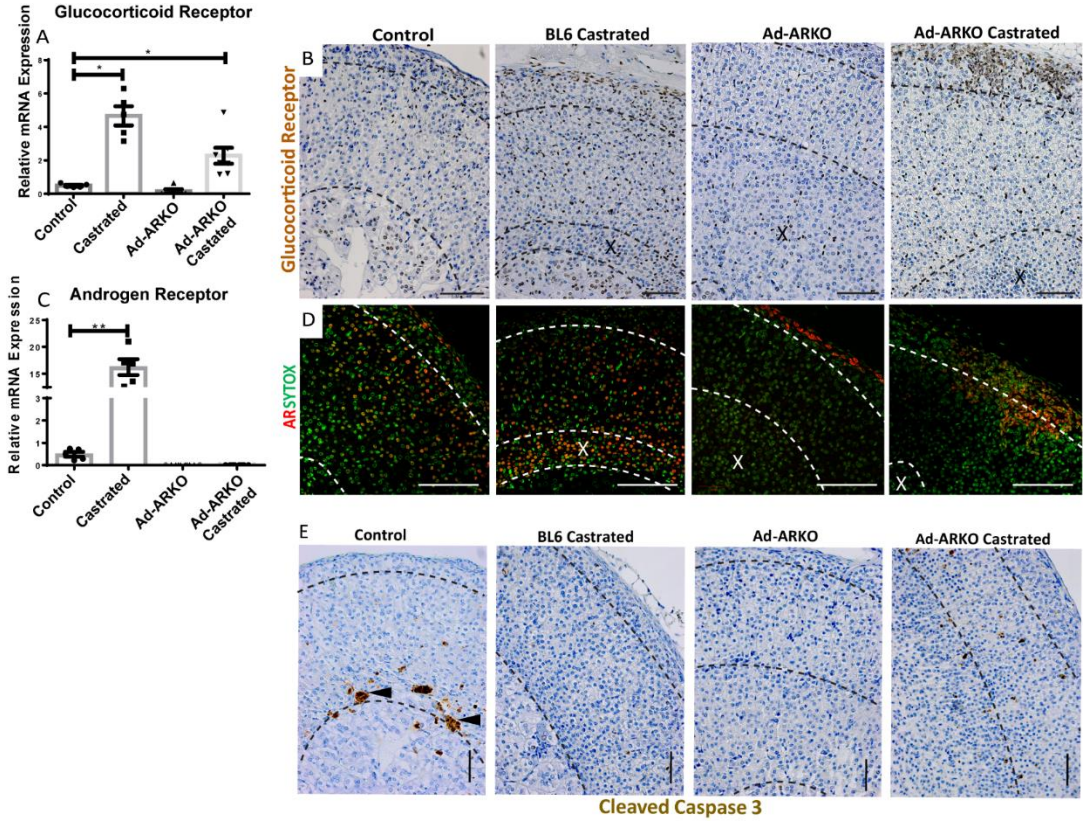


Figure 3-12. Ad-ARKO Castrated mice display cell clusters in the adrenal capsule. ***(A)*** *Glucocorticoid receptor gene expression is significantly upregulated in cohorts following castration (one-way ANOVA; n=7-8 * p<0.05, Tukeys post-hoc analysis, error bars SEM)* ***(B)*** *GR immunostaining shows increase localisation in cohorts following castration, clusters of cells expressing GR can also be observed at the zona glomerulosa of Ad-ARKO castrated mice. N=5.* ***(C)*** *Androgen receptor gene expression has a significant upregulation in castrated males (one-way ANOVA;** p<0.001, Tukeys post-hoc analysis, error bars SEM).* ***(D)*** *AR immunostaining (Red) shows an increase in expression in the X-zone region of the cell with a loss of expression in the ZG. Clusters of AR positive cells in the ZG can be observed in Ad-ARKO Castrated mice. Green: sytox counterstain. N=5.* ***(E)*** *Cleaved caspase 3 immunostaining shows cell death at the cortex medulla boundary. No cleaved caspase staining can be observed in Bl6 castrated mice or Ad-ARKO animals. In Ad-ARKO castrated mice cell death can be seen throughout the cortex. N=5. Scale Bars 50µm. Annotations; M=medulla, X= X-zone.*

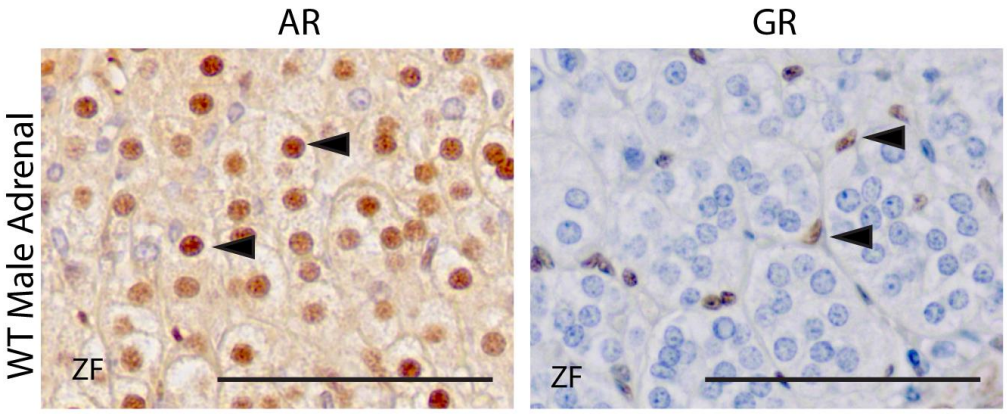


Figure 3-13. Immunostaining reveals AR and GR are not localised in the same cell populations. *AR localisation in the WT male adrenal does not occupy the same cell types that localise. Scale Bars 50µm. Arrows highlight the different cell types expressing AR (large circular nuclei of steroidogenic cells) and GR (small spindle shaped nuclei located between the steroidogenic cells). Annotations; ZF= zona fasciculata,*

3.2.11 Aged Ad-ARKO mice show increased disruption to adrenal cortex.

A study investigating *Prkar1a* gene ablation in the adrenal cortex noted the presence of an X-zone and that a Cushing's Syndrome phenotype develops when the mice were aged to 12 months (12m) (285). Due to the presence of an X-zone in all experimental cohorts, in addition to the downregulation of *Prkar1*, (Fig. 3-7D) a cohort of Bl6 castrated, Ad-ARKO and Ad-ARKO castrated mice were aged to 12 months to see if a similar phenotype or additional disruption to the adrenal cortex can be seen following prolonged loss of AR or circulating androgens.

Comparison of adrenal weights from d80 to 12m showed no changes in Bl6 castrated and Ad-ARKO castrated cohorts following prolonged loss of circulating androgens but a significant increase in adrenal weight was observed in 12m Ad-ARKO mice compared to d80 Ad-ARKO mice (Fig. 3-14A). Consistent with this, interrogation of adrenal morphology revealed no obvious visual difference in structure in 12m controls compared to d80 controls (Fig. 3-14B). Prolonged loss of circulating androgens in 12m Bl6 castrated mice also results in no major disruption to the adrenal cortex compared

to d80 Bl6 castrated mice (Fig. 3-14B). Likewise, analysis of 12m Ad-ARKO castrated mice show no disruption the adrenal cortex compared to d80 Ad-ARKO castrated mice (Fig. 3-14B), however the X-zone is significantly smaller at 12m than at d80 and is breaking down (Fig. 3-14A). Despite this, in accordance with the increase in adrenal weight, a worsening of cortex disruption is observed in 12m Ad-ARKO animals compared to d80 Ad-ARKO mice (Fig. 3-14B). Large spindle cell lesions can be observed in the adrenal capsule, that extend down into the ZF. Additionally, 12m Ad-ARKO mice have an X-zone that is significantly larger than the X-zone that is observed in d80 Ad-ARKO mice (Fig. 3-15A). Analysis of 12m Ad-ARKO X-zones also reveals enlarged hypertrophic eosinophilic cells (Fig. 3-15C). Circulating corticosterone remains significantly increased in 12m Ad-ARKO males compared to 12m controls, similar to that observed at d80 (Fig.3-15B). Similarities can be drawn between the phenotype observed in 12m Ad-ARKO mice and *Prkar1a* KO mice, such as enlarged, mislocated X-zone, presence of cell clusters in the outer cortex and elevated corticosterone. This highlights a potential relationship between AR and *Prkar1a1* in adrenal cortex regulation. Together these data show that, whilst chronic loss of androgens does not exacerbate the phenotype established earlier in life, continued AR signalling is essential to prevent age-related degeneration of the adrenal cortex associated with further X-zone expansion and the development of spindle cell lesions.

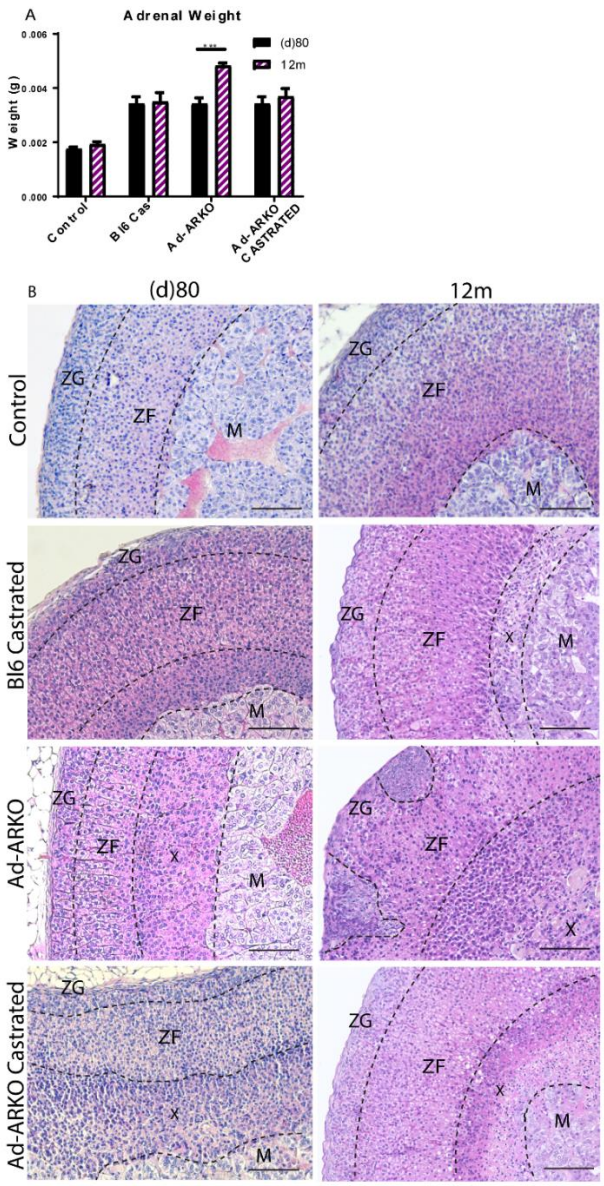


Figure 3-14. Aged Ad-ARKO mice display large cell clusters in capsule and large eosinophilic cells (A) *Adrenal weight analysis showed a significant increase in 12m Ad-ARKO mice compared to (d)80 Ad-ARKO mice (two-way ANOVA; n=5-8 *** p<0.0001, Tukeys post-hoc analysis, error bars SEM), and no weight changes are observed in any other cohort.* ***(B)*** *Morphology analysis of 12m old Bl6 castrated, Ad-ARKO and Ad-ARKO castrated mice reveals in 12m cohorts with no circulating androgens show no major cortex disruption and X-zone regression. 12m Ad-ARKO mice have severe cortex disruption, with presence of large spindle cell lesions in the outer cortex (highlighted by dotted lines) and an enlarged X-zone. d80 cohorts have n=5, 12m cohorts have n=3. Annotations; M=medulla, ZF= zona fasciculata, ZG = zona glomerulosa, X= X-zone.*

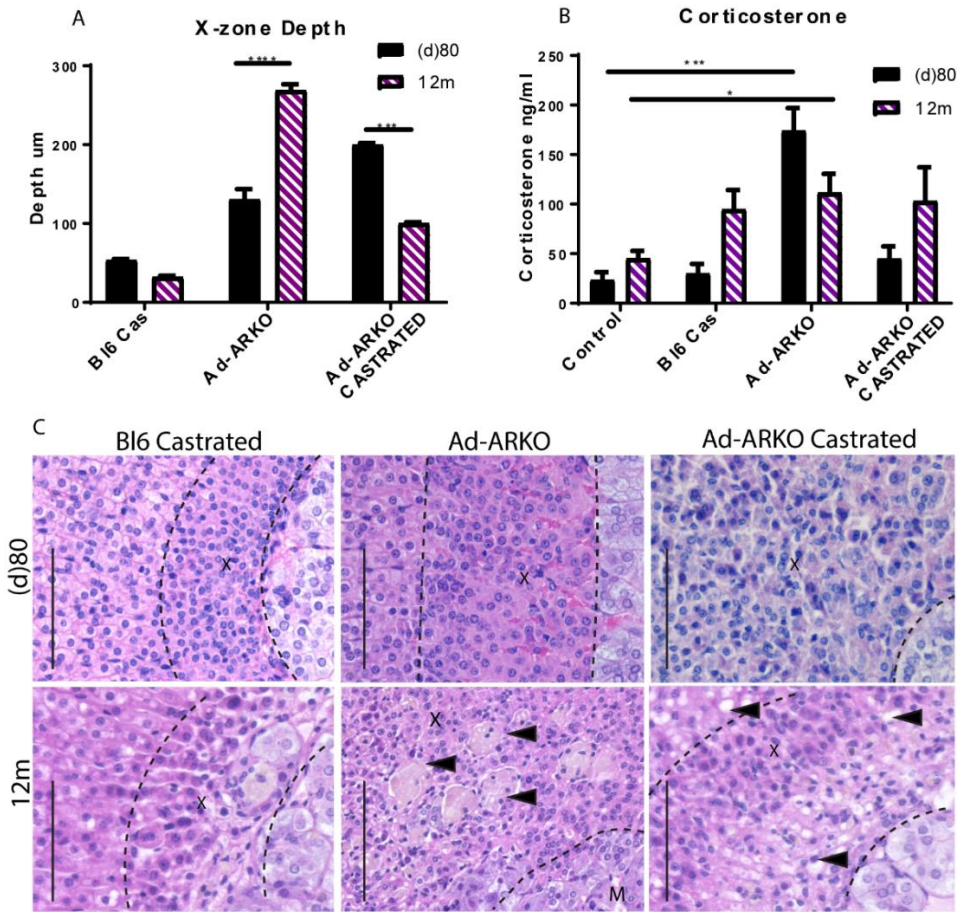


Figure 3-15. Aged Ad-ARKO mice X-zones are larger than those in early adulthood. (A) *Measurement of X-zone depth show a significant increase in 12m Ad-ARKO mice compared to (d)80 Ad-ARKO mice (two-way ANOVA; n=5-8 **** p<0.0001, Tukeys post-hoc analysis, error bars SEM). In contrast 12m Ad-ARKO castrated mice show a significant decrease in X-Zone depth when compared to (d)80 Ad-ARKO mice (two-way ANOVA; n=5-8 **** p<0.0001, Tukeys post-hoc analysis, error bars SEM).* ***(B)*** *Circulating corticosterone analysis display no significant difference between (d)80 and 12m cohorts, however, 12m Ad-ARKO mice have a significant increase in corticosterone compared to 12m controls (two-way ANOVA; n=5-8 * p<0.05, Tukeys post-hoc analysis, error bars SEM).* ***(C)*** *Interrogation of X-zone morphology revealed X-zone regression via shrinkage and vacuolisation when compared to d80 Bl6 castrated and Ad-ARKO castrated mice. 12m Ad-ARKO mice show large multinucleated cells (denoted by arrows) in the X-zone and enlargement compared to d80 Ad-ARKO mice. N=5. Scale bars 50µm.*

3.3 Discussion

To investigate the role of androgen receptor signalling in the adrenal a novel mouse model with a specific ablation of androgen receptor in the adrenal cortex was used, compounded with additional manipulation of circulating androgen levels. Results show AR expression in the human and mouse adrenal and highlight that the mouse is a viable model to investigate androgen signalling in the adrenal cortex. Results demonstrate that androgen signalling exclusively via AR is required for X-zone regression during puberty. Cortex measurements define differences in X-zone morphology depending on whether circulating androgens or AR have been targeted, suggesting androgens and androgen receptor are working both together and independently of each other in different adrenal processes. This result is further strengthened by loss of AKR1B7 localisation in models following removal of circulating androgens but not AR which still maintained AKR1B7 positive cells. Prolonged loss of circulating androgens in aged mice reveal apparent adrenal feminisation with eventual X-zone regression. However, aged animals with AR ablation reveal severe cortex disruption, spindle cell hyperplasia and X-zone expansion. These data together show AR signalling is required to facilitate X-zone regression and mediate circulating androgen action on the adrenal cortex, with loss of AR leading to excessive stimulation of the adrenal and development of X-zone expansion and spindle cell lesions.

Investigation of androgen receptor signalling in the developing mouse adrenal highlighted that AR expression can be observed as early as e13.5, this expression is maintained into adulthood and is present in all cortical zones. AR is not only observed in developing mouse adrenal, it is also expressed in the human second trimester foetal tissue. Results also show expression of AR in the adult human adrenal. Localisation of AR in the human adrenal has previously never been shown. This may be because previous research has focussed on androgen production and its impacts thereafter, however little research has focused on the autocrine regulation of AR in the adrenal itself, despite the fact that the adrenal has been shown to react to the loss of androgens through castration by the development of an X-zone (23, 56). However, the mechanism

underpinning this and its importance remains unexplored. By describing the localisation in human and mouse, this highlights that not only can the rodent be used as a suitable model to explore androgen action within the adrenal but AR is important in the control of adrenal development and function. An addition issue with examining human adrenal AR may be due to the difficulty of detection of AR at a protein level. During the course of trying to localise AR in human samples involved the use of numerous antibodies until one was found that worked. Due to there being no literature detailing AR localisation, where AR would be localised would be unclear. To ensure the antibody has worked, additional human foetal and adult testis controls were used. Interestingly, in human adrenal the localisation appears to be cytoplasmic.

To provide a comprehensive overview of the impact of androgens on the adrenal cortex, it was important to ascertain the impacts not only of the loss of androgens but also to establish if androgens had the potential to over stimulate the adrenal cortex or impact the X-zone and its morphology. Following a single or chronic dose of hCG, examination of adrenals revealed no changes in cortex morphology in controls or in any experimental cohorts. Additionally, there appears to be no impact on X-zone morphology in any of these cohorts. For these reasons, animals treated with hCG were not taken any further as part of this study. This data highlights that there may be an upper limit to circulating androgen action on the adrenal cortex.

Confirmation of AR ablation from steroidogenic cells in the adrenal cortex was achieved via AR immunostaining. Initial investigation of the adrenal reveals an increase in adrenal weight from d35 and maintained into adulthood in Ad-ARKO mice, this coincides with the presence of an additional cortical zone with characteristics of the rodent X-zone. This data demonstrates that expression of AR in the adrenal cortex is essential during puberty for regression of the X-zone (34).

Androgens and AR can work together and independently of each other to regulate gene expression (273, 288, 201), so to fully investigate the impact of androgen signalling on the adrenal, additional models were added to the study, these include Bl6 castrated (in which adrenal AR was present but not circulating testosterone) and Ad-ARKO castrated mice (in which neither adrenal AR or circulating testosterone were present).

All cohorts have increased adrenal weight in adulthood compared to controls, and morphology analysis and staining with 20 alpha-HSD (281) show that they have all developed X-zones. This suggests that androgens binding to AR is required for appropriate removal of X-zone cells from the cortex during development, and that AR or testosterone cannot act independently to promote X-zone regression. Analysis of the X-zone morphology highlights that the X-zones appears to be denser and occupy different proportions of the cortex depending on whether androgens or androgen receptor have been disrupted, suggesting that not only is AR signalling through AR to regulate the cortex zonation but they are acting independently of each other as well. Cortex measurements confirmed significant morphological differences between cohorts. Measurements highlighted differences in X-zone cell density and X-zone depth. This result further strengthened the argument for androgen AR independent signalling in the adrenal. To explore these differences in more detail and determine potential mechanisms that androgen or AR controlled, markers for stress, development and steroidogenesis were investigated.

Investigation of circulating corticosterone, a key indicator of adrenal stress response (282, 289), shows that Ad-ARKO animals have an increase in circulating corticosterone, that resolves upon castration. This potentially suggests that castration is rescuing the increased stress response from the adrenal induced by AR ablation. Circulating androgens target numerous tissues and the impact of castration has been demonstrated in the hypothalamus and its release of gonadotropin releasing hormone (GnRH) (290) and also can exert feedback directly at the level of the pituitary and impact LH secretion (291). This disruption to organs that can regulate the adrenal cortex may also play a role. When analysing corticosterone, the handling of the animals must be considered. Prior to the collection of the animals, minimal stress must be achieved to obtain true basal levels. Upon handling, there is only a brief window (2-5 minutes) before circulating corticosterone becomes elevated (292). Attempts were made to cull the animal as quickly and efficiently as possible from the initial cage handling to being placed in the CO_2 box. However, this handling process may not have been the most efficient practice. Animals were not separated and in instances where there was a struggle to catch the animal, all gives opportunity for corticosterone levels

to be elevated. The handling procedure aforementioned has resulted in minor stress to the mice and elevated corticosterone levels. For true basal levels a more refined method would need to be used. This could be achieved by leaving animals in a quiet room prior to collection and separating into individual cages to lessen the contact time for the animals. Other culling methods such as decapitation over CO_2 has been suggested to be preferential due to the quick nature of the process. However, it can be argued that all conditions the mice were collected under are relative to the controls. Despite having corticosterone levels at the level deemed 'minor stress', all mice were collected under the same conditions so are relative to the controls.

Due to the early expression of AR, it was important to investigate the potential impact of androgen ablation throughout development. Any literature regarding androgens and their regulation of the X-zone have determined androgen action does not occur until puberty, it was thought that once circulating testosterone reached high enough concentration this would ultimately lead to X-zone regression (95). However, investigation of 20 alpha-HSD staining throughout postnatal development shows that X-zone cells are already more developed in size and abundance compared to controls at d12, d21 and d35. This tells us, contrary to the literature, that this regulation of the adrenals by androgens is occurring before puberty and that androgens are potentially positively regulating these foetal cells during development. Essential regulators of the adrenal cortex, *Sf1* and *Dax1* (57, 59), were investigated in d80 adrenals to determine if disruption to androgen signalling impacted these genes. Results show a significant up regulation in *Sf1* transcript in all experimental cohorts compared to controls. It has previously been shown that androgens are a repressor of *Sf1* activity (293), this is an important mechanism to regulate *Sf1* and its target genes. The expression of *Sf1* is a tightly regulated process during development (293), sustained elevated levels of *Sf1* could have serious implications for the developing cortex and a number of diseases are associated with elevated *Sf1* expression, such as childhood adrenocortical tumours (82). Removal of AR and circulating androgens resulted in an increase in *Dax1* expression. *Dax1* is important for the differentiation of stem and progenitor cells located in the adrenal capsule (294, 295), this increase in expression could suggest that following the complete loss of both androgens and AR signalling, adrenal cortex cell

differentiation is affected. Due to the ectopic maintenance of an X-zone in androgen signalling depletion models, investigation of transcript levels of *Prkar1* was carried out. This gene has previously been shown to regulate X-zone cells and when ablated resulted in a condition known as Primary Pigmented Adrenocortical Disease (PPNAD) (29). Results highlight that there is a significant downregulation in its transcript levels in all experimental cohorts. There is little literature to suggest that AR could be upstream of *Prkar1a*, so to say that AR is directly regulating this gene would be an over simplification. However, there is some literature that describes AR signalling working in congress with *Prkar1* in driving castration resistant prostate cancer (142). These results combined, show an important regulatory role for androgen signalling in the adrenal cortex during development and adult cortex maintenance and highlights relationships between key adrenal regulators and AR signalling.

To assess if the loss of androgen signalling impacted steroidogenesis, a panel of steroidogenic enzyme genes were investigated. *Cyp21a1* is involved with the biosynthesis of the steroid hormones aldosterone and corticosterone and is usually found in ZF cells (15). Results revealed an increase in Cyp21a1 expression and CYP21 localisation throughout the entire adrenal cortex following loss of androgen signalling. An abundance of literature can be found detailing the impacts of loss of function or mutated *Cyp21* but there is little literature detailing the impacts of overexpression in the adrenal cortex, further analysis into would be needed to ascertain the direct impact, however, it could lead to overactive steroidogenesis in the adrenal glands.

3β-HSD is essential in the biosynthesis of steroid hormones in the adrenal gland (284), and is expressed throughout the adrenal cortex. Protein localisation of 3β-HSD shows varied staining in the experimental cohorts. X-zones in Bl6 castrated animals stained positive for 3βHSD, this suggests potentially that these cells have come from a steroidogenic lineage. However, 3βHSD localisation in Ad-ARKO mice showed sporadic positive cells with Ad-ARKO castrated animals showing no staining for 3βHSD in the X-zone. The presence of 3β-HSD positive cells in the X-zone of Ad-ARKO mice could potentially be X-zone cells that have differentiated from foetal cells to steroid producing cells, this differentiation could potentially be driven by continued signalling from androgens and following castration this could explain why no positive

cells are observed in Ad-ARKO castrated mice. AKR1B7 is a well-established ZF marker (40) that is integral for detoxifying the adrenal cortex from toxic by-products arising from cholesterol cleavage (296). Due to change in cortex composition, it was important to assess how this impacted *Akr1b7* expression. Results highlights no changes in transcript levels however in both castrated experimental cohorts in which androgens are removed, no AKR1B7 positive cells can be observed in the cortex. However, Ad-ARKO mice still express AKR1B7 in the adrenal cortex. This data along with the changes in morphology observed in the X-zone, shows that androgens can act independently of their receptor to mediate gene expression in the adrenal cortex. AKR1B7 has previously been shown to be regulated by androgens in the vas deferens (297), however it was thought its regulation in the adrenal cortex was under the regulation of *Sf1* (298). The data suggests that this is potentially not the case, that either androgens are regulating AKR1B7 or are working with *Sf1* to regulate its expression.

Due to the known relationship between AR and GR (162, 205), analysis of the impact of AR ablation on GR expression in experimental cohorts was investigated. Investigation of GR signalling in the adrenal cortex revealed a significant increase in GR transcript in cohorts following castration. Protein localisation reveals clusters of GR-positive cells in the outer cortex in Ad-ARKO castrated mice which also stained positive for AR, however, AR and GR are not normally expressed in the same cell types in the adrenal cortex, suggesting the presence of an abnormal cell population that potentially may be undifferentiated cells. Adrenocortical cells under normal conditions migrate towards the cortex-medulla boundary and undergo apoptosis (53). Due to failed clearance of X-zone cells and the development of cell clusters in the cortex, investigation of apoptosis marker cleaved caspase 3 was used to determine if the cortex was still able to appropriately clear cells from the cortex. Protein localisation of Cleaved caspase 3 shows no positive cells in BL/6 castrated and Ad-ARKO mice, suggesting that following loss of androgen signalling the adrenal does not show normal patterns of cell death. This phenotype has been documented before in a global AR knockout model, however, this was thought to be a result of loss of signalling from the pituitary (162), results demonstrate that this is potentially not the case and that adrenal-specific androgen receptor signalling is responsible is the driving this phenotype.

The majority of this study focused on animals in early adulthood or short-term loss of circulating androgens, so the final part of the study investigated the implications of chronic loss of androgen signalling on the adrenal cortex. The study investigating *Prkar1* ablation in the adrenal cortex and the noted the presence of foetal cell did not detect a phenotype until aged (29). To determine if chronic loss of androgens resulted in a similar or more severe phenotype, mice were aged to 12 months. Investigation of morphology revealed significant disruption to the adrenal cortex of 12m Ad-ARKO mice with significant enlargement of the X-zone, which also presented with large eosinophilic cells throughout. A similar large multinuclear cell population was also observed in 6m Leydig cell ARKOs, which were shown to be large infiltrating macrophages. Staining with cleaved caspase could help determine if these enlarged cells are adrenocortical cells or are infiltrating macrophages. Furthermore, large cell clusters in the outer cortex were present that protruded into the centre of the cortex. These cell types are characteristic of spindle cell hyperplasia 'type A' cells (299).

Morphology and X-zone measurements show that X-zone regression is occurring in long term castrated cohorts, with no disruption to the remainder to the cortex outside of that which could be expected with age. X-zone morphology and regression observed in 12m Ad-ARKO castrated animals is typical of what would be observed under normal conditions in an aged WT female (34). The X-Zone in WT females is maintained into adulthood, and regresses at first pregnancy, however if there is no pregnancy, the X-zone eventually regresses with age via vacuolization (89). However, in 12m Ad-ARKO mice there is severe disruption to the cortex and a progressive phenotype observed. Elevated circulating corticosterone is also maintained in 12m Ad-ARKO mice, highlighting a sustained stress response. Prolonged periods of corticosterone excess is known to be involved in a number of clinical conditions such Cushing's syndrome (300), cardiovascular disease (301) and decreased bone density (302). Together this data demonstrates that prolonged loss of circulating androgens does not progress the phenotype observed in d80 castrated mice, and that potentially the adrenal has become feminized. The adrenal following loss of AR only, results in a large spindle cell lesions and X-zone expansion and maintained elevated corticosterone. Throughout this chapter, the results demonstrate that androgens can act

both with and independently of their receptor, prolonged exposure of the adrenal cortex to androgens without AR could potentially lead to off target activation of signalling pathways by androgens leading to X-zone expansion and spindle cell hyperplasia.

In conclusion, this analysis highlights numerous targets of androgen signalling within the adrenal cortex. Not only are androgens signalling through their receptor essential for the regression of the X-zone during puberty, but that they can work together and independently to modulate adrenal function. Prolonged loss of androgens results in a phenotype similar to a virgin female adrenal, however loss of AR alone results severe cortex disruption with an increase in X-zone size, development of hyperplasia and maintained elevated stress response. Additionally, these results highlight that despite the mouse adrenal not producing androgens, androgen action plays a key functional role in the adrenal gland.

Chapter 4: Loss of androgen signalling via AR results in increased spindle cell hyperplasia in the female mouse adrenal

4.1 Introduction

Androgens signalling through androgen receptor (AR) have been well characterised in the development of male specific phenotypes, including embryogenesis, spermatogenesis (164, 303), sexual behaviours (304) as well as maintaining fertility throughout life (305), however the role of androgen signalling has also been shown to be integral in the female, during ovarian folliculogenesis (306), embryonic implantation (307) and uterine and breast development (308). Androgens in the female are secreted by the adrenal and the ovary and can also be formed peripherally, particularly from adrenal DHEAS (114). In women the adrenals account for approximately 25% of circulating androgens (309), with cross sectional analyses revealing that decreased circulating androgens impact health and wellbeing in young and aging women (114, 310). The primary focus of adrenal androgen research has been on the disruption to production of androgens from the adrenal cortex with scarcely any research covering the role of adrenal AR signalling itself. This could be in part due to the perceived lack of suitable rodent model, as the mouse adrenal does not produce androgens (111).

Despite this, treatment with testosterone in both males and females have shown to impact the mouse adrenal X-zone (34, 36, 311). The X-zone in the mouse adrenal is sexually dimorphic and regresses differently in males and females. Both the male and female mouse adrenal develops an X-zone during postnatal development and as previously described, involution occurs during puberty in males and at first pregnancy in females. If no pregnancy occurs, regression occurs eventually over time with age (34, 38). Historical studies in female mice show that the X-zone regresses following treatment with testosterone-propionate, suggesting that androgens are able to regulate the female adrenal cortex (36). Studies that investigated the effects on the adrenal following the loss of circulating androgens through gonadectomy noted the re-

development of the X-zone and in certain strains, the development of adrenocortical tumours were more frequent in females (56).

Adrenal masses can be detected in approximately in 4% of the population, with the majority of these originating from the adrenal cortex region (312). It is thought that adrenocortical tumours (ACT) may result in aberrant steroid production and in cases where tumours become cancerous, lead to high mortality rates due to tumour growth (312, 313). Studies investigating the mechanisms of adrenal tumour development have advanced greatly through the use of appropriate technology to dissect the relationship between tumour development and the potential genes involved in its progression (22, 285, 312). However, despite adrenocortical cancer being rare, its mortality rates are high and little is known about the underlying mechanisms driving its progression from benign to cancerous. A main precursor for the development of ACTs in humans and in rodents is the development of spindle cell hyperplasia (299). Some of these studies also noted that the phenotype observed in females was often more severe than in males (285, 287, 299). Certain mouse strains, IQI/Jic mice in particular, have been used to investigate the development of spindle cell hyperplasia as they are thought to help elucidate the underlying mechanism driving tumour progression (299, 314).

For these reasons, adrenal androgen receptor knockout females were investigated to determine if the loss of AR impacts female adrenal cortex development, if it has the potential to regulate the X-zone in the female during pregnancy and if loss of AR leads to the development of ACTs. Results show that, despite previous literature, AR is dispensable for the postnatal developing female adrenal and during X-zone regression following pregnancy. Furthermore, results highlighted that following disruption to adrenal AR, the development of spindle cell hyperplasia could be seen in young adult females that progressed with age. These result point to dysfunction androgen signalling as a possible mechanism in the development of spindle cell hyperplasia.

Aims 4.1.1

- To determine if ablation of androgen receptor impacts the postnatal developing female adrenal
- To determine if loss of androgen receptor impacts adult cortex morphology, function and stress response
- To determine if Ad-ARKO females develop spindle cell lesions
- To establish if loss of androgen receptor results in failed X-zone regression during pregnancy

4.2 Results

4.2.1 Confirmation of ablation of androgen receptor from the female adrenal

Because AR is X-linked, first generation Ad-ARKO females are in fact mosaic for presence/absence of AR expression (due to random X-chromosome inactivation), whilst the F2 animals completely lack adrenal AR from steroidogenic adrenocortical cells. Both mosaic and total Ad-ARKO females are used in this study as partial ablation enables investigation of autocrine AR action in the adrenal cortex in addition to the effects of complete loss of adrenal AR (Fig. 4-1A). AR is expressed in all zones of the adrenal cortex in littermate controls, in contrast, partial ablation of AR from steroidogenic adrenocortical cells can be seen in 1st Generation Ad-ARKO females and complete ablation from steroidogenic adrenocortical cells in 2nd Generation Ad-ARKO females (Fig. 4-1B). These data confirm the ablation of AR from the adrenal cortex, and that this model permits tissue-specific interrogation of androgen signalling in the female mouse adrenal.

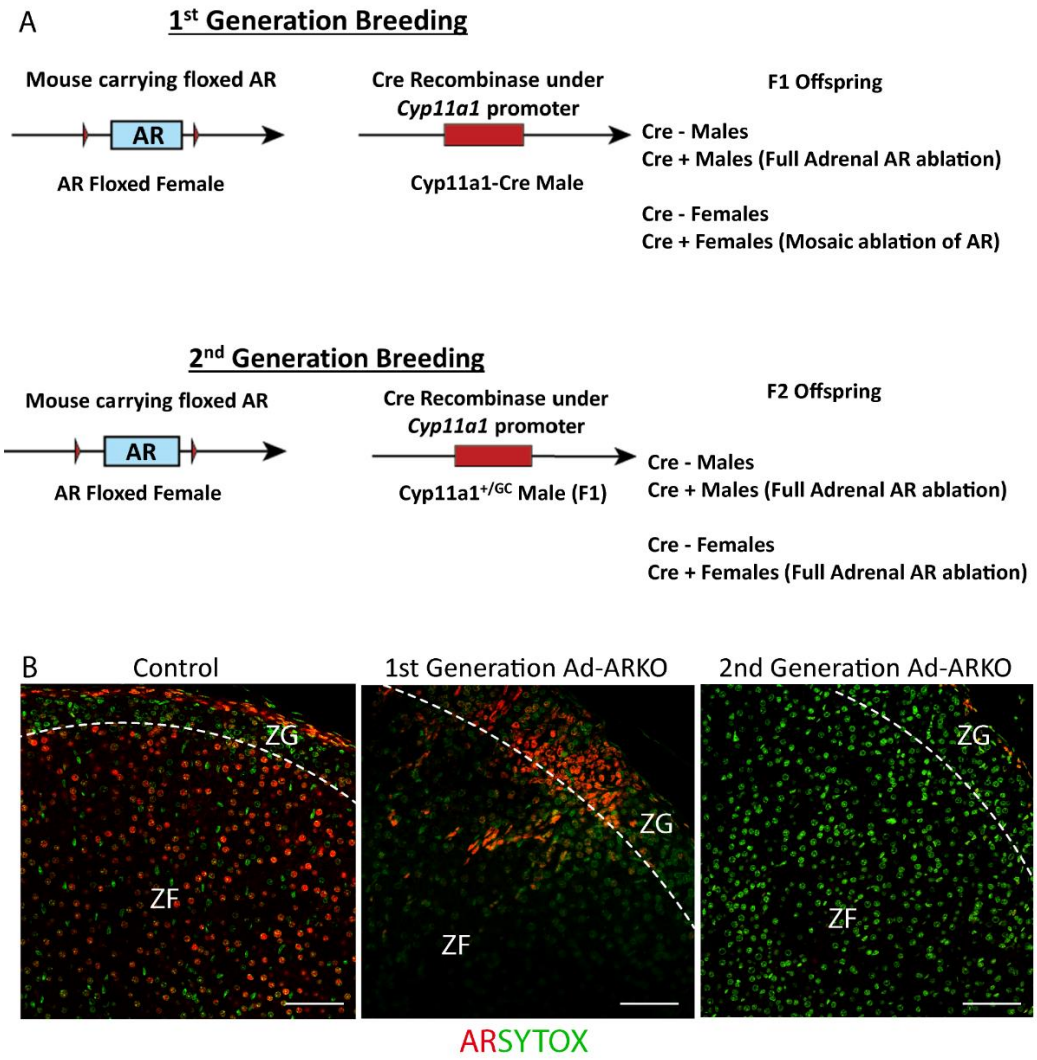


Figure 4-1. Breeding strategy and confirmation of AR ablation in the adrenal cortex. (A) *Schematic detailing breeding strategy for Ad-ARKO females. AR is X-linked so requires two rounds of breeding to achieve a complete adrenal AR knockout female. 1st generation offspring are generated by breeding an AR floxed female to a male carrying the Cyp11a1-Cre. 2nd generation breeding uses an AR floxed female and an AR knockout male from the F1 offspring.* ***(B)*** *Immunostaining of AR in the adrenal cortex reveals partial ablation in the 1st Generation Ad-ARKO and complete adrenal AR ablation in the 2nd generation Ad-ARKO females. N=5. Red: androgen receptor protein, Green: sytox counterstain. Scale Bars 50µm. Annotations; ZF= zona fasciculata, ZG = zona glomerulosa.*

4.2.2 Analysis of population ratios reveals significantly fewer 2nd Generation Ad-ARKO females

General observations made when breeding 2nd generation Ad-ARKO females noted that there appeared to be fewer of them being born. To address this, a Chi squared analysis was performed (315). Results showed the expected 50/50 distribution between 1st generation Cre- and Cre+ males and females (Fig. 4-2A). Analysis of 2nd generation litters revealed a normal distribution of 50/50 between Cre+ and Cre- males, however, this was not observed in 2nd generation females. 79% of 2nd generation females are Cre- with only 21% of females carrying complete ablation of adrenal AR (Fig. 4-2B). Due to the small numbers of 2nd Generation females that were born during the study, analysis using these mice was limited to a d80 cohort and a pregnancy cohort only. These results demonstrate that following complete adrenal AR ablation, either fewer 2nd Generation Ad-ARKO females are being born or they are not surviving after birth.

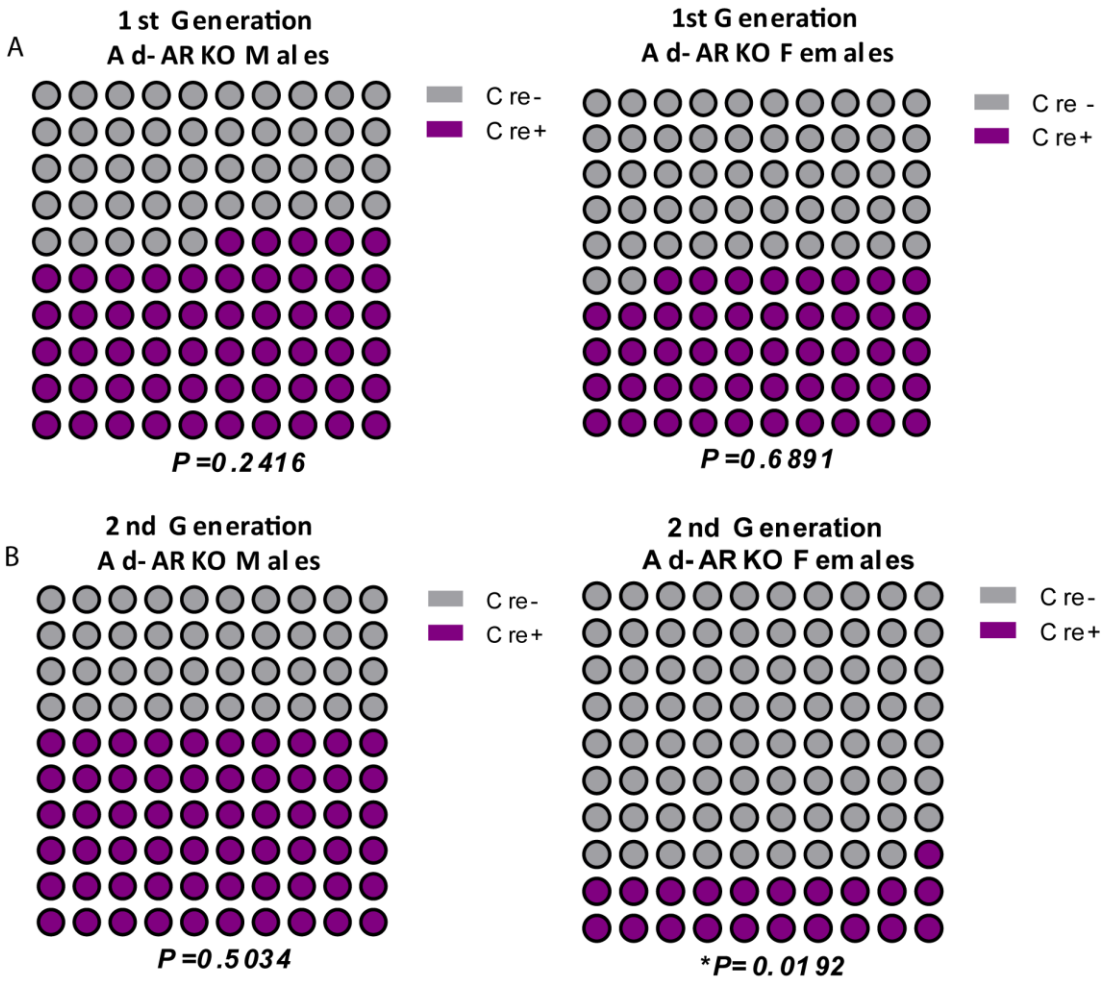


Figure 4-2. Significantly fewer 2nd Generation Ad-ARKO females are born. ***(A)*** *Chi squared analysis of 1st Generation Ad-ARKO observed and expected litter ratios revealed 50% Cre- and 50% Cre+ in males (X^2 P=0.2416) and females (X^2 P=0.6891).* ***(B)*** *Chi squared analysis of 2nd Generation Ad-ARKO observed and expected litter ratios revealed 51% Cre- and 49% in males (X^2 P=0.5034) and 79% Cre- and 21% Cre+ in females (X^2 *P=0.0192), showing significantly fewer Cre+ females being born.*

4.2.3 Large lesions are observed in the outer cortex of 1st Generation Ad-ARKO females

To establish whether there is any impact on the developing adrenal following AR ablation, investigation of time points postnatal day (d)12, d21, d35 and d80 were performed. No changes in body weight could be detected at any of the time points investigated when compared to age matched controls (Fig. 4-3A). Analysis of adrenal weight revealed no changes compared to controls at d12, d21 or d35, but adrenal weight is significantly increased in 1st Generation Ad-ARKO females at d80 when compared to littermate controls (Fig. 4-3B). Histological analysis reveals no differences in the postnatal developing adrenal when compared to littermate controls, but at d80 large lesions in the adrenal cortex of 1st Generation Ad-ARKO females are observed (Fig. 4-4A). Together these data demonstrate that loss of AR does not impact the female postnatal developing adrenal at d12, 21 or d35, with a phenotype not being observed until d80. For these reasons, the only time point analysed was d80. Additionally, these results demonstrate the development of lesions and disruption to the adrenal cortex following loss of AR in early adulthood.

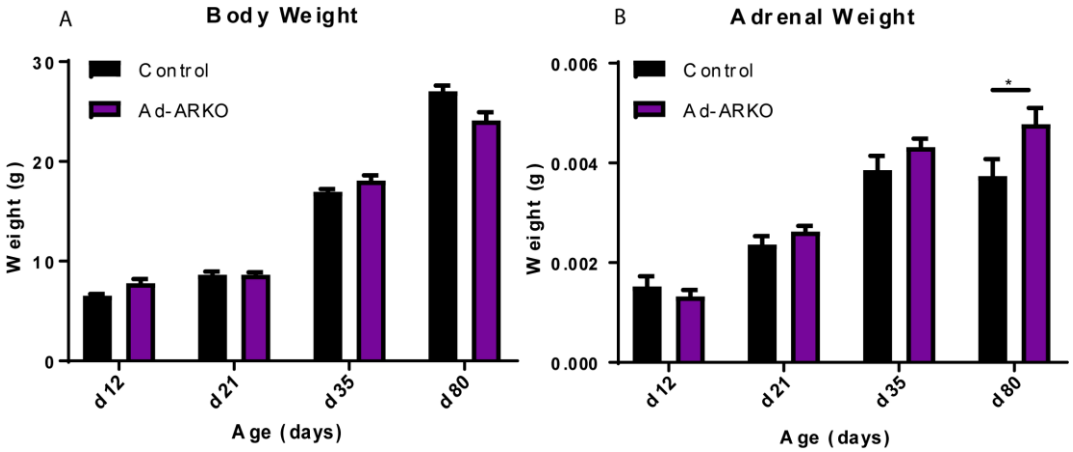


Figure 4-3. 1st Generation Ad-ARKO show increased adrenal weight at d80. (A) *Interrogation of body weights at d12, d21, d35 and d80 revealed no changes in body weight in 1st Generation Ad-ARKO females compared to WT controls (Two-way ANOVA, Tukeys post-hoc analysis, error bars SEM), N=8.* ***(B)*** *Interrogation of adrenal weights at d12, d21, d35 revealed no changes compared to WT controls. A significant increase in adrenal weight is observed at d80 compared to WT controls. (Two-way ANOVA, Tukeys post-hoc analysis, error bars SEM), N=8.*

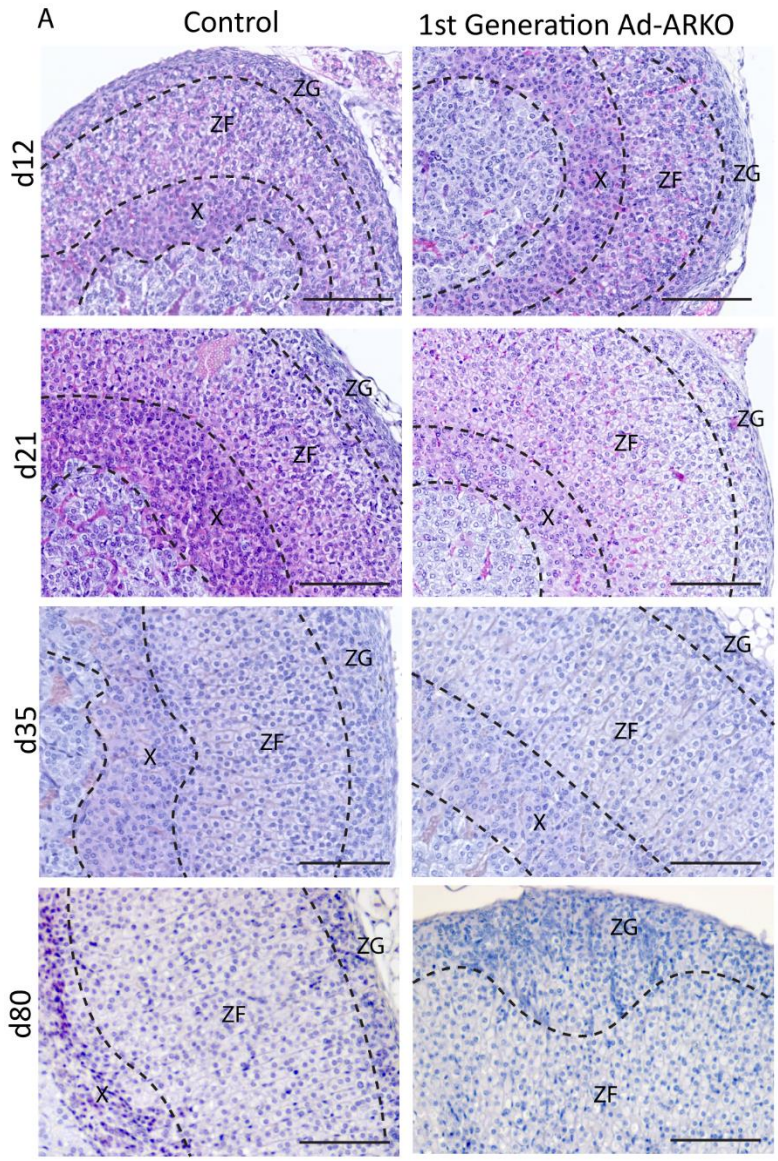


Figure 4-4. 1st Generation Ad-ARKO show no differences during development but display large cell clusters in the outer cortex. (A) *H&E analysis of adrenal morphology show no differences at d12, d21, d35 compared to WT controls. At d80 large cell clusters can be observed in the outer adrenal cortex of 1st Generation Ad-ARKO females. N=5. Annotations; ZF= zona fasciculata, ZG = zona glomerulosa, M=medulla. Scale Bars 100µm.*

4.2.4 Partial and complete ablation of adrenal AR results in spindle cell hyperplasia and X-zone vacuolization

To investigate adrenal disruption in more detail, analysis of d80 littermate controls, 1st Generation Ad-ARKO and 2nd Generation Ad-ARKO was performed. Body weight measurements reveal no changes in either cohort when compared to littermate controls (Fig. 4-5A). Adrenal weight was increased in both 1st Generation and 2nd Generation Ad-ARKO females compared to littermate controls (Fig. 4-5B). Histological analysis of both experimental cohorts revealed significant disruption to the outer cortex compared to littermate controls. Upon closer inspection, morphology of the cell types that make up the abnormal cell clusters in the outer capsule are characteristic of spindle cells with their ovoid or spindle shape (287), classically named 'type A cells'(287, 299). Another, but rarer form of spindle cell hyperplasia can also occur termed 'type B cells', which are polygonal in shape and can vary in size. Morphological analysis show no B type cells are present (287). Spindle cells can be seen in both 1st and 2nd Generation Ad-ARKO females, with 2nd Generation Ad-ARKOs displaying a progressive phenotype from 1st to 2nd generations and presence of spindle cells throughout the majority of the outer cortex (Fig. 4-5C). Spindle cell hyperplasia is not uncommon in aging mice and has been noted more commonly in females than in males, however, the development of these cells in young mice is extremely rare (280, 299).

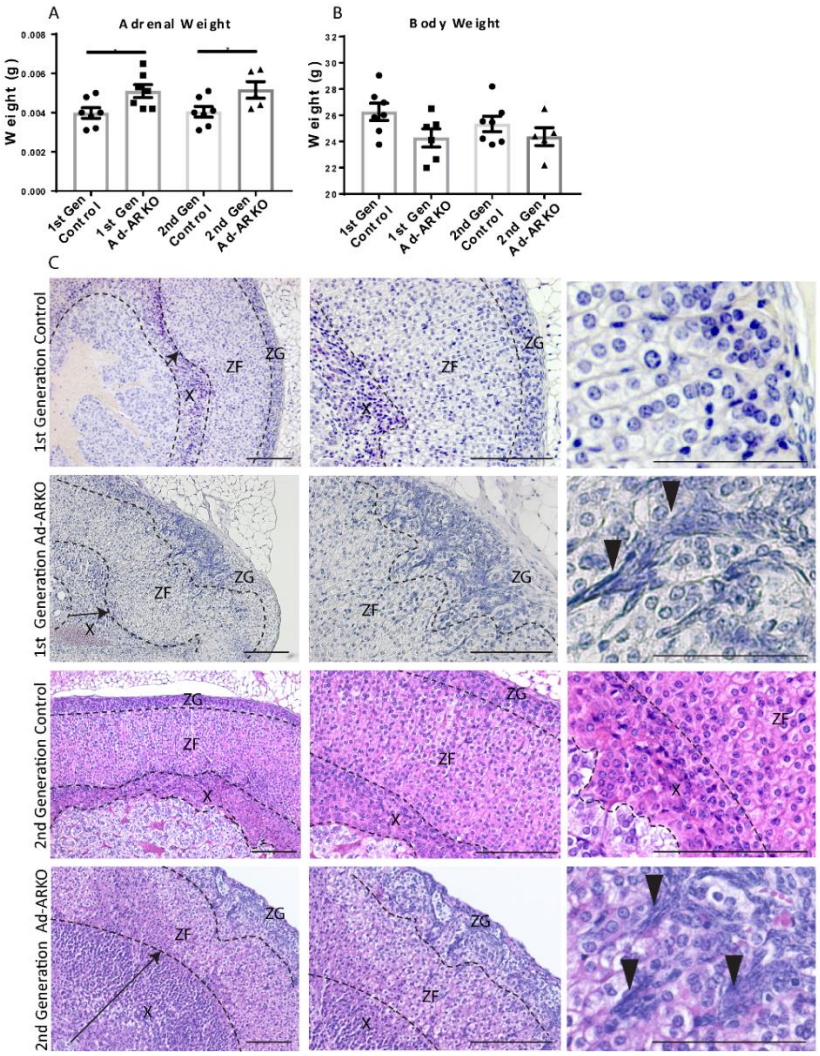


Figure 4-5. Spindle cell lesions can be observed in 1st and 2nd Generation Ad-ARKO females. ***(A)*** *Bodyweight analysis of d80 WT controls, 1st Generation Ad-ARKO and 2nd Generation Ad-ARKO females revealed no changes.* ***(B)*** *Adrenal weight analysis shows a significant increase in 1st and 2nd Generation Ad-ARKO mice compared to respective controls (one-way ANOVA; n=5-8, *p<0.05, *p<0.05, Tukeys post-hoc analysis, error bars SEM).* ***(C)*** *H&E analysis reveals spindle cell lesions in the outer adrenal cortex that protrude down from the ZG into the ZF in 1st Generation Ad-ARKO females. These spindle cell lesions can also be observed in 2nd Generation Ad-ARKO females but are more progressive and occupy a larger portion of the adrenal cortex. Spindle cells denoted by arrows. N=5. Annotations; ZF= zona fasciculata, ZG = zona glomerulosa, M=medulla. Scale Bars 100µm.*

Due to the potential for spindle cells to occur naturally it was important to determine if the presence of these cells increased following partial or complete adrenal AR ablation. 20% of littermate controls at d80 had spindle cell hyperplasia, increasing to 60% of 1st Generation Ad-ARKO females d80 and a further increase to 80% in 2nd Generation Ad-ARKOs at d80 (Fig. 4-6A). The X-zone in the female adrenal is maintained in adulthood, but is kept in a well-defined region at the cortex-medulla boundary (34, 95). Interrogation of X-zone morphology revealed expansion of foetal cells from the X-zone through the cortex in 1st and 2nd Generation Ad-ARKOs. Again, this phenotype is visually more pronounced in the 2nd Generation Ad-ARKO females. In addition to the development of spindle cell hyperplasia, pockets of vacuolization can also be seen in X-zones of both experimental cohorts following AR ablation, which is not observed in littermate controls, presence of vacuoles suggests damage and potential breakdown of the adrenal cortex (Fig. 4-6B). Together this data demonstrates the early onset and increase in occurrence of spindle cell hyperplasia as a result of adrenal AR ablation. Body weight, adrenal weight and adrenal morphology of 1st and 2nd generation controls show no differences, so from this point on both 1st and 2nd generation Ad-ARKO mice are compared to the 1st Generation control group, termed 'controls'.

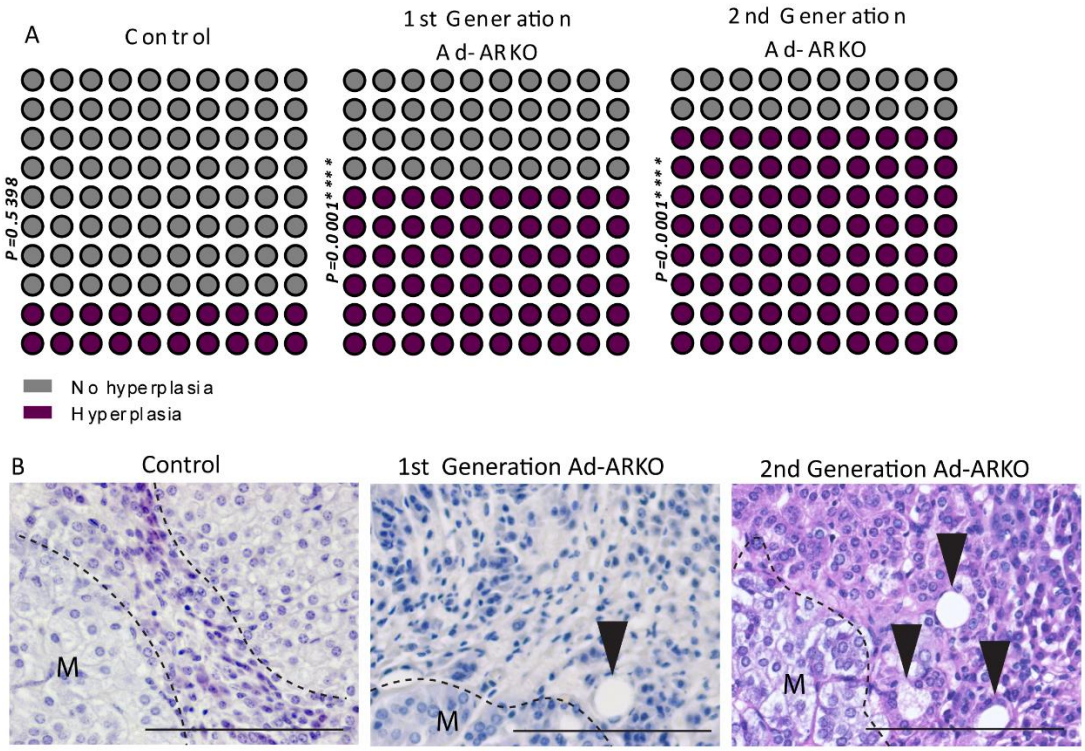


Figure 4-6. There is an increase in spindle cell occurrence in 1st and 2nd generation Ad-ARKO females. ***(A)*** *Interrogation of spindle cell development in Ad-ARKO females reveals a 60% occurrence in 1st Generation Ad-ARKO females (X^2 P=0.0001) that increases to 80% in 2nd Generation Ad-ARKO females (X^2 P=0.0001) compared to only a 20% occurrence in WT controls (X^2 P=0.5398), (n=5). For statistical analysis, observed vs. expected values were obtained from morphology analysis in WT controls (80% no hyperplasia and 20% hyperplasia), these were then used for the statistical analysis on 1st and 2nd generation Ad-ARKO mice.* ***(B)*** *Analysis of X-zone revealed expansion and migration up the cortex, with pockets of vacuolization throughout in 1st Generation Ad-AKRO females. The same but more progressive phenotype can also be observed in 2nd Generation Ad-ARKO females when compared to controls. Scale Bars 100µm. Annotations; M=medulla.*

4.2.5 Ablation of AR results in disruption to cortical markers 20 alpha-HSD and AKR1B7

To determine if cortex disruption observed in Ad-ARKO females impacted stress response or resulted in disruption to cortical markers, analysis of the stress biomarkers corticosterone (316) and *Mc2r* (317) was performed. In addition 20-α-hydroxysteroid dehydrogenase (20 alpha-HSD), a well described X-zone marker (281), and Aldo-keto reductase family 1, member 7 (AKR1B7) a well-defined zona fasciculate marker known for detoxifying cholesterol cleavage products was also analyzed (298). No changes are observed in circulating corticosterone or *Akr1b7* transcript (Fig.4-7A, B) but a significant increase in 20alpha-HSD is observed (Fig. 4-7C). Analysis of 20 alpha-HSD immunostaining highlights disruption to the X-zone with migration of foetal cells through the cortex (Fig. 4-8A). AKR1B7 immunostaining showed disruption in both 1st Generation and 2nd Generation Ad-ARKO females with large portions of the adrenal cortex no longer positive for AKR1B7 (Fig. 4-8B). Together these data demonstrate that ablation of AR in the adrenal does not impact the adrenal response to stress, however, spread of foetal cells through the cortex and loss of AKR1B7 could have serious implications for adrenal function, as the adrenal may no longer be able to deal with toxic products from steroidogenesis.

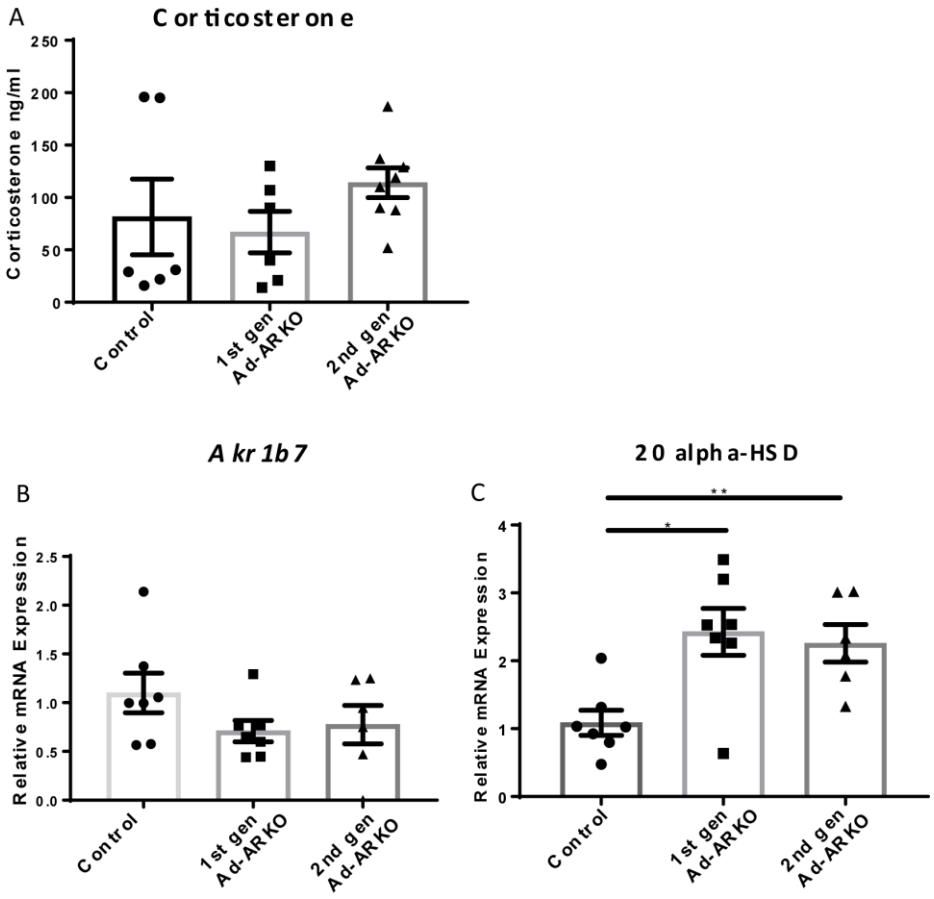


Figure 4-7. AR ablation results in no changes in the adrenals stress response. ***(A)*** *Analysis of serum corticosterone revealed no changes in 1st or 2nd Generation Ad-ARKO females when compared to controls.* ***(B)*** *Investigation of Akr1b7 transcript revealed no changes when compared to compared to controls* ***(C)*** *Investigation of 20alpha-HSD transcript revealed a significant increase in expression in 1st and 2nd Generation Ad-ARKO females when compared to controls (one-way ANOVA; n=8, *p<0.05, **p<0.001, Tukeys post-hoc analysis, error bars SEM).*

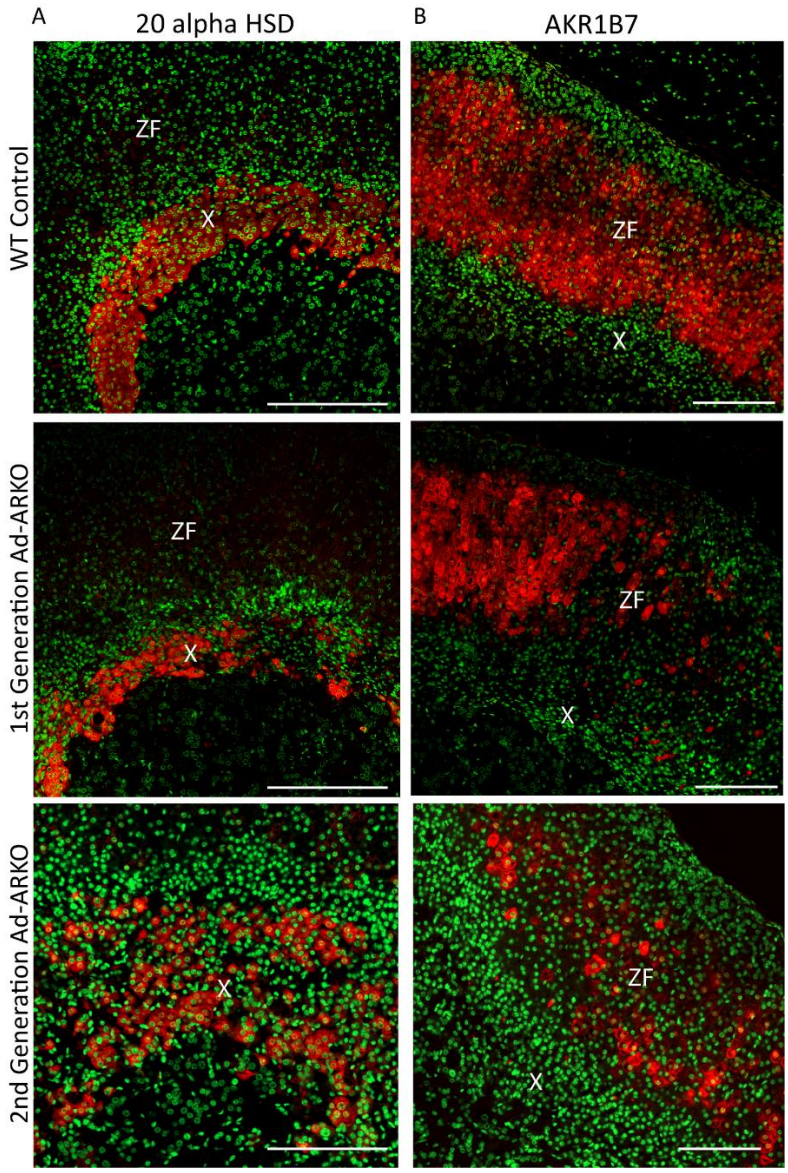


Figure 4-8. AR ablation results in disruption to adrenocortical markers. ***(A)*** *Immunostaining of 20alpha-HSD revealed migration of foetal cells in 2nd generation Ad-ARKO females up through the cortex, compared to WT controls were the X-zone is maintained at the cortex medulla boundary. Red: 20alpha-HSD protein, Green: sytox counterstain. N=5.* ***(B)*** *Immunostaining of AKR1B7 revealed loss of positive cells throughout the adrenal cortex in 1st and 2nd Generation Ad-ARKO females. Red: AKR1B7 protein, Green: sytox counterstain. N=5. Scale Bars 100µm. Annotations; ZF= zona fasciculata, X=X-zone.*

4.2.6 Spindle cells in the adrenal cortex are positive for GR

As the adrenal plays an integral role in steroidogenesis (280, 318), a panel of steroidogenic enzymes and additional steroid receptors were analyzed for any disruption. Changes in protein immunolocalization of steroid cell marker 3βHSD were observed (319). Littermate controls have 3βHSD positive cells in the ZG and ZF, analysis of 1st and 2nd Generation ARKO females revealed that spindle cells are not steroidogenic and do not stain positive for 3βHSD (Fig. 4-9A). Protein localization of GR showed that the spindle cells that have developed in the outer cortex are positive for GR (Fig 4-9B). Due to the substantial portions of the adrenal being occupied by spindle cells in Ad-ARKO females, this could suggest that large parts of the adrenal are now no longer producing steroids and normal adrenal function is impaired. Additionally, over expression of GR in ACTs and has been used as a marker to help distinguish between benign and malignant ACTs (320).

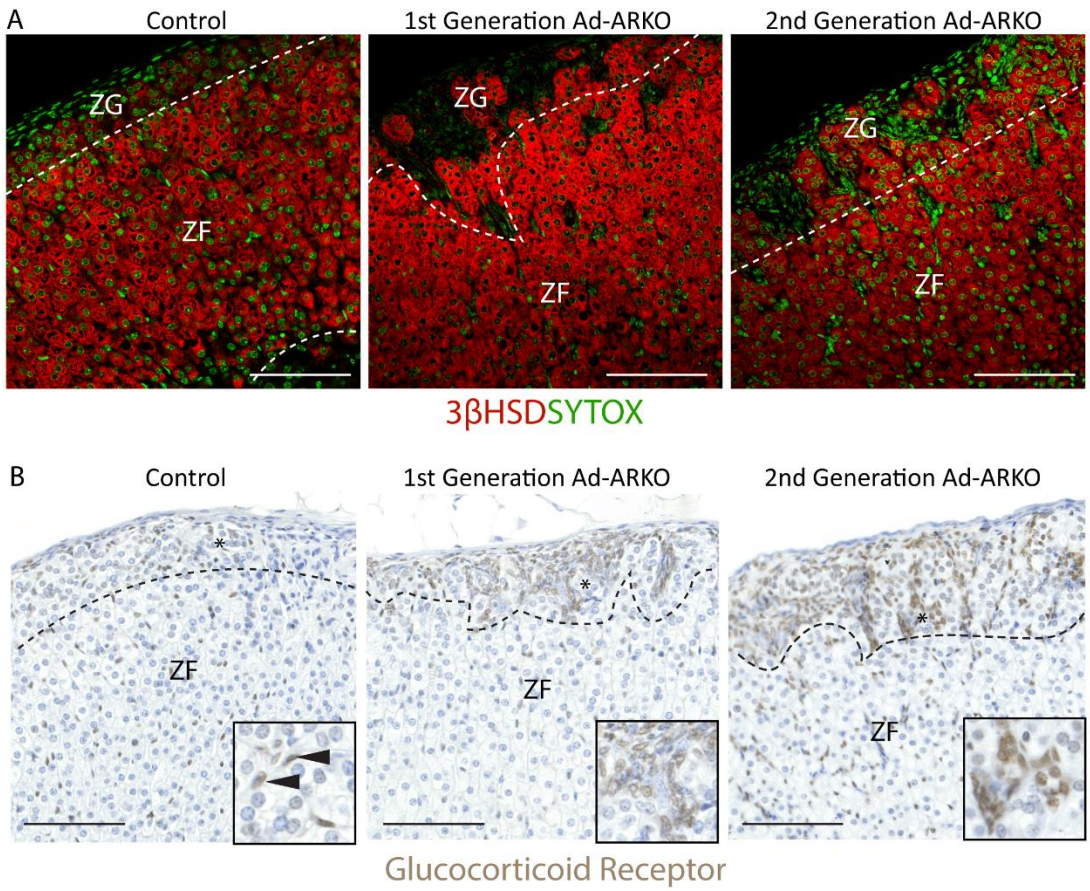


Figure 4-9. Spindle cell lesions are positive for GR and lack 3βHSD expression. ***(A)*** *Immunostaining of 3βHSD localisation revealed that the spindle cells do not express 3βHSD in 1st or 2nd Generation Ad-ARKO females, compared to WT controls that are positive in both the ZG and ZF. Red: 3βHSD protein, Green: sytox counterstain. N=5.* ***(B)*** *Immunostaining of GR revealed that spindle cells are positive for GR in 1st and 2nd Generation Ad-ARKO females. (*) denotes insert location. N=5. Scale Bars 100µm. Annotations; ZF= zona fasciculata, ZG= zona glomerulosa.*

4.2.7 Fewer proliferating cells are observed in Ad-ARKO females

The balance between cell proliferation and cell death is essential to maintain healthy tissue (2, 15). As spindle cell hyperplasia is present in Ad-ARKO adrenals, proliferation marker PCNA (321) and apoptosis marker cleaved caspase 3 (322) were analyzed. Cell clearance from the adrenal cortex under normal conditions occurs at the cortex medulla boundary (2), immunostaining of cleaved caspase 3 showed no changes in location or frequency of apoptosis in any experimental cohort compared to littermate controls (Fig. 5-10A). Proliferation and differentiation of cells in the outer cortex is essential for appropriate population and maintenance of the adrenal cortex zones (323); immunostaining and cell counts revealed significantly fewer proliferating cells in 1st and 2nd Generation Ad-ARKOs compared to littermate controls (Fig. 4-10B). Fewer PCNA positive cells suggests that the normal proliferation and differentiation of the adrenal cortex is perturbed following loss of AR (Fig. 4-10B, C). It has been noted that in the presence of spindle cells, there is an increase in infiltrating mast cells into the adrenal cortex (299), for these reasons analysis of *C-kit* transcript, a specific marker for mast cells was analyzed (324). Results show a decrease in *C-kit* transcript (Fig. 4-10D) which suggests that there is not an increase but a decrease in mast cell infiltration to the adrenal cortex.

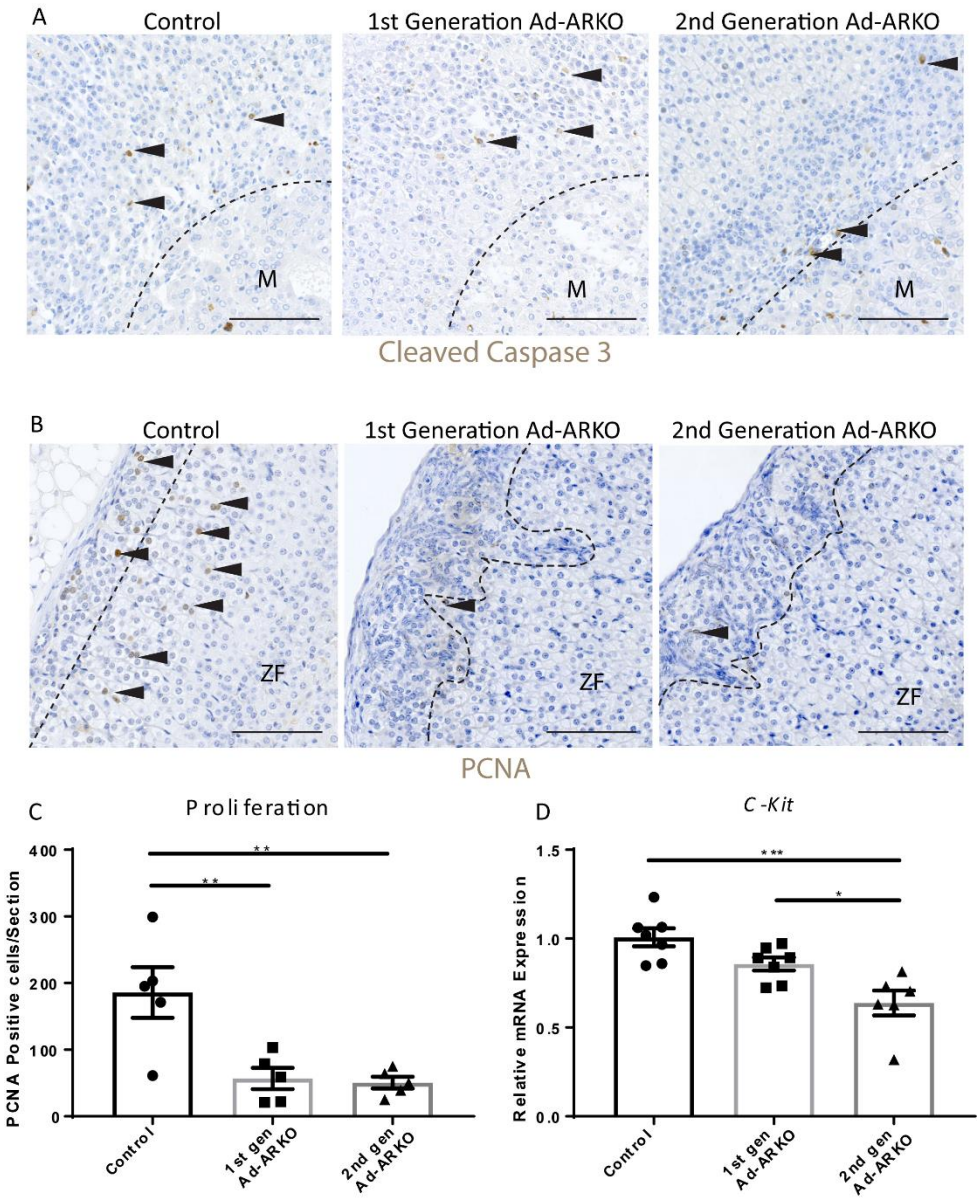


Figure 4-10. Fewer proliferating cells are observed on 1st and 2nd Generation Ad-ARKO females compared to WT controls. ***(A)*** *Investigation of cleaved caspase localisation revealed no changes in either 1st or 2nd Generation Ad-ARKO females when compared to WT controls.* ***(B, C)*** *Immunostaining of PCNA localisation and cell counts reveal fewer proliferating cells in 1st Generation and 2nd Generation Ad-ARKO females compared to WT controls. N=5* ***(D)*** *Transcript analysis of C-kit showing a significant downregulation compared to controls (one-way ANOVA; n=7-8, *p<0.05, ***p<0.001, Tukeys post-hoc analysis, error bars SEM). Scale Bars 100µm. Annotations; M=medulla, ZF= zona fasciculata.*

4.2.8 Progression of spindle cell hyperplasia and X-zone expansion can be observed in aged 1st Generation Ad-ARKOs

Due to changes observed in X-zone cells in d80 Ad-ARKOs, examination of these foetal cells was carried out to establish if they regressed as normal with age or if they continued to expand throughout the cortex and to ascertain whether the spindle cell hyperplasia progresses with age, so a 12 month old (12m) aged cohort of 1st generation Ad-ARKOs was analyzed. Analysis of body weights revealed no change in 1st Generation Ad-ARKO females when compare with 12m littermate controls (Fig. 4-11A). A significant decrease in adrenal weight is observed in 1st Generation Ad-ARKO females (Fig. 4-11B). There is no significant difference in circulating corticosterone observed between 12m littermate controls and 12m 1st Generation Ad-ARKOs (Fig. 4-11C).

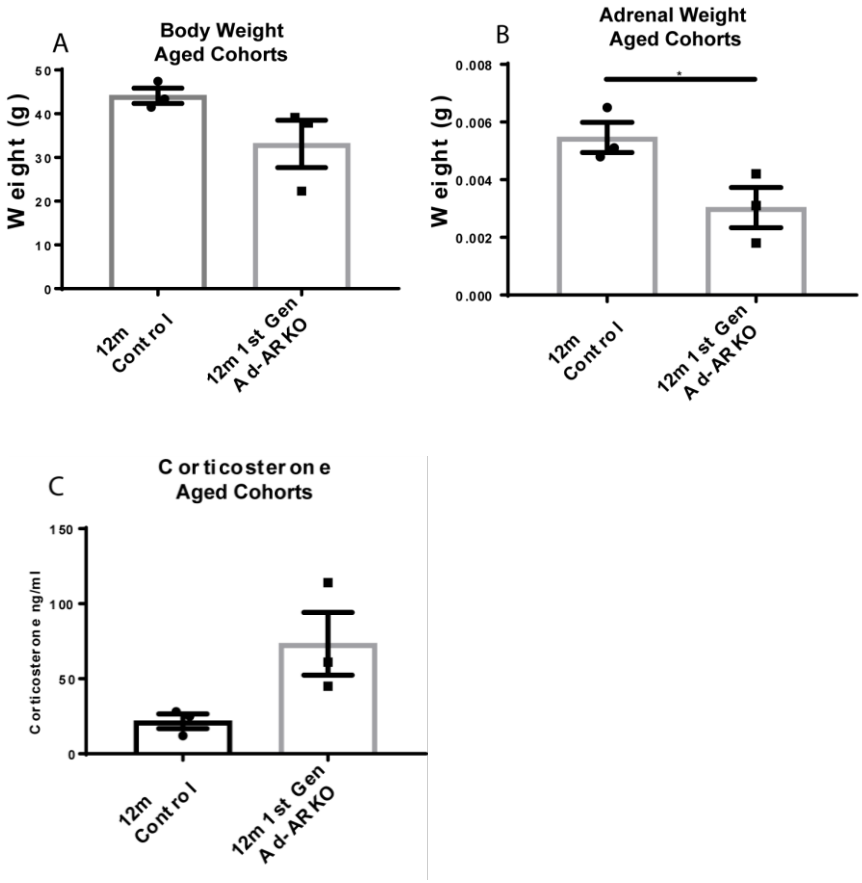


Figure 4-11. 12 month Ad-ARKO 1st Generation females have decreased adrenal weight. ***(A)*** *Analysis of body weight revealed no changes in 12m 1st Generation Ad-ARKO females compared to 12m WT controls.* ***(B)*** *Analysis of adrenal weight revealed a significant decrease in 12m 1st Generation Ad-ARKO females when compared to age matched controls (one-way ANOVA; n=3, *p<0.05, *p<0.05, Tukeys post-hoc analysis, error bars SEM).* ***(C)*** *Analysis of serum corticosterone revealed no changes in 12m 1st Generation Ad-ARKO females compared to aged matched WT controls.*

It is known that aging littermate females can develop spindle cell hyperplasia (299), therefore the aim was to establish if the occurrence of these cells increased with age in Ad-ARKO females and how that compares to the development of spindle cells in littermate females. Interrogation of morphology revealed 12m littermate controls had developed spindle cell hyperplasia that was mainly localized to the outer capsule. In 1st Generation Ad-ARKO females however, spindle cells protrude down into the ZF and occupy a large proportion of the outer cortex (Fig. 4-12A). In addition to this, X-zone regression usually occurs in aging littermate females that have not had a pregnancy (34, 89), and this regression was indeed observed in 12m littermate control females. However, the X-zone in nulliparous 1st Generation Ad-ARKO females does not regress with age and has in fact become more disrupted, with observed migration further into the cortex (Fig. 4-12B). This X-zone expansion and increase in spindle cells leaves very little ZF left in the cortex. Presence of spindle cells in the cortex increases from 20% of d80 littermate females to 80% of 12m littermate females, and from 60% of d80 1st Generation Ad-ARKO females to 100% of 12m Ad-ARKO females (Fig. 4-12C). Although the occurrence of spindle cells does increase in littermate females with age, becoming similar to 1st generation Ad-ARKOs, spindle cells appear more severe and X-zone expansion is noted. This data highlights that loss of AR results in progressive spindle cell hyperplasia and X-zone expansion in aged female mice.

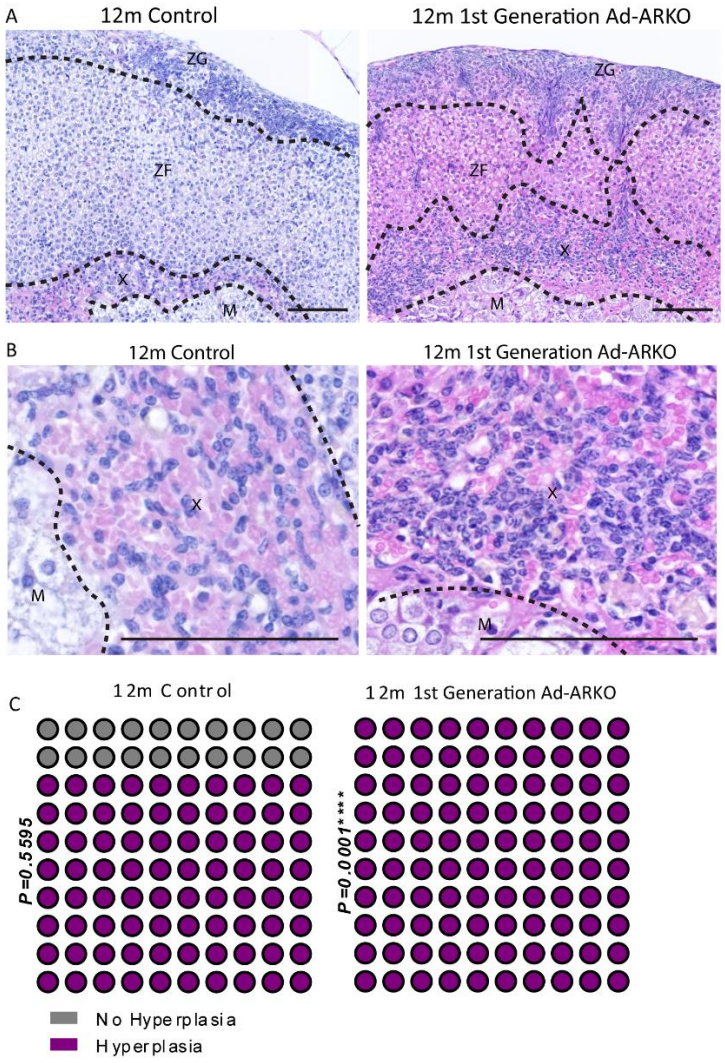


Figure 4-12. 12 month Ad-ARKO 1st Generation females reveals progressive spindle cell hyperplasia. ***(A)*** *Morphology analysis reveals spindle cell development in 12m WT controls and is maintained in the ZG region of the cortex. The same lesions can be observed in 12m 1st Generation Ad-ARKO females, however, lesions are more extensive.* ***(B)*** *Analysis of X-zone morphology in 12m old cohorts revealed X-zone regression in WT controls as expect, but X-zone expansion can be seen in 12m 1st Generation Ad-ARKO females with progression up through the adrenal cortex with minimal ZF remaining in the cortex.* ***(C)*** *Occurrence of spindle cells in 12m WT females increases to 80% (X^2 P=0.5.398) and 12m 1st Generation Ad-ARKO females have spindle cell hyperplasia in 100% of samples analysed (X^2 P=0.0001). N=5. Scale Bars 100µm. Annotations; ZF= zona fasciculata, ZG = zona glomerulosa, M=medulla, X=X-zone.*

4.2.9 AR is dispensable for X-zone regression during pregnancy in the female adrenal

Results in chapter 3 show that AR is required for X-zone regression during puberty in male mice (Fig. 3-4). Due to failed X-zone regression in Ad-ARKO males and X-zone expansion and migration through the cortex in ageing female Ad-ARKOs, it was important to investigate if loss of AR resulted in failure of the normal X-zone regression during pregnancy. Interrogation of morphology reveals normal X-zone regression in both parous littermate controls and parous 2nd Generation Ad-ARKOs. (Fig. 4-13A). Immunostaining of cleaved caspase reveals apoptosis of the X-zone in both parous littermate controls and parous 2nd Generation Ad-ARKOs (Fig. 4-13B). Immunostaining for 20 alpha-HSD confirms removal of X-zone cells with only a few positive cells remaining in both parous littermate controls and 2nd Generation Ad-ARKOs (Fig. 4-13C). Despite no changes in X-zone regression being observed between littermate controls and Ad-ARKOs during pregnancy, circulating corticosterone is significantly elevated in parous 2nd Generation Ad-ARKO females compared to parous littermate controls (Fig. 4-13D). These results demonstrate that, in contrast to males, AR is dispensable for X-zone regression in females, but results in an elevated stress response after pregnancy.

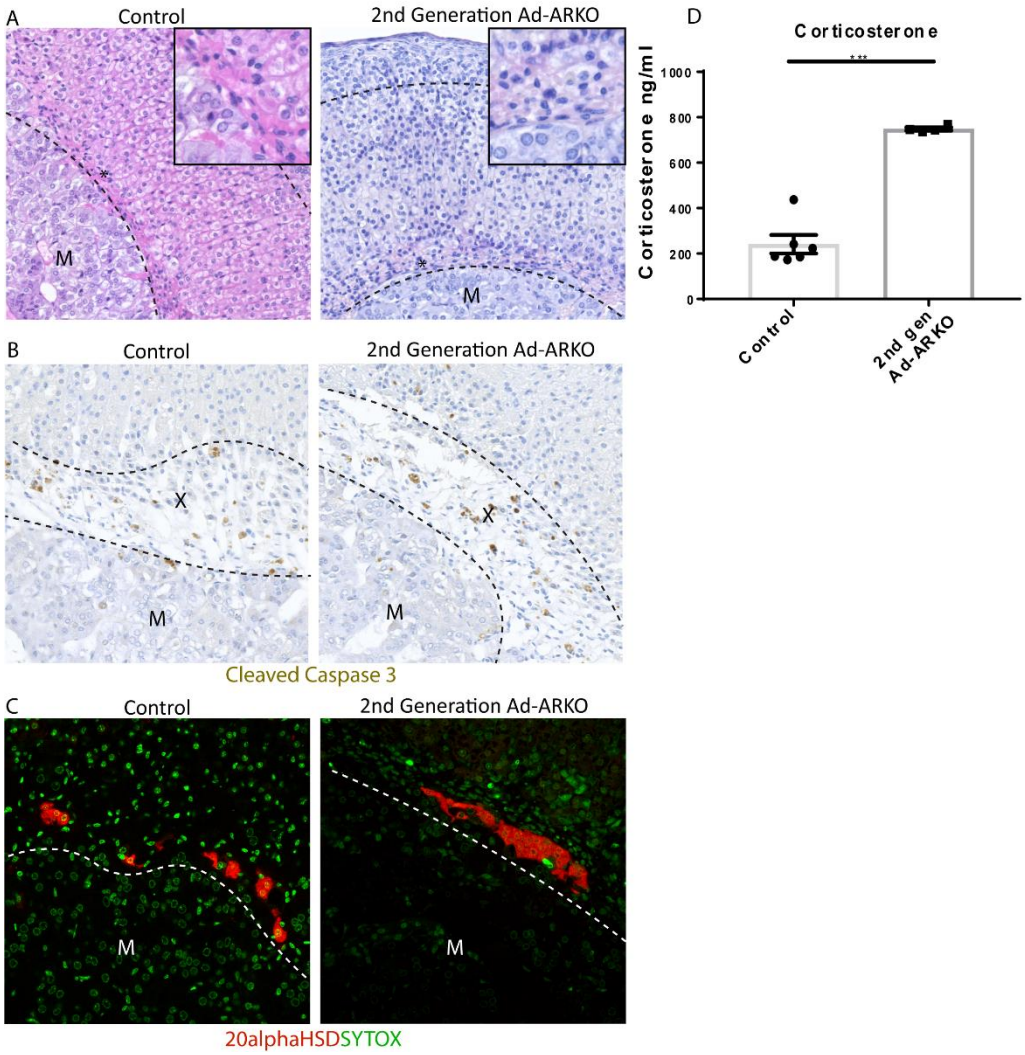


Figure 4-13. AR is dispensable for X-zone regression during pregnancy. ***(A)*** *Analysis of X-zone morphology following a single pregnancy in WT and 2nd Generation Ad-ARKO females reveals regression of the X-zone in both cohorts. N=5.* ***(B)*** *Immunostaining of cleaved caspase 3 shows apoptosis of the X-zone in both WT and 2nd Generation Ad-ARKO females. N=5.* ***(C)*** *Immunostaining of 20 alpha-HSD localisation shows only a few X-zone cells remaining in the cortex of WT controls and 2nd Generation Ad-ARKO females. Red: 20alpha-HSD protein, Green: sytox counterstain. N=5.* ***(D)*** *Analysis of circulating corticosterone shows a significant increase in 2nd Generation Ad-ARKO females following pregnancy (Students t-test; n=5-6), *p<0.05, *p<0.05, error bars SEM) compared to WT controls. (*) denotes insert location. Scale Bars 100µm. Annotations; M=medulla, X=X-zone.*

4.3 Discussion

Through the use of the *Cyp11a1*-Cre, ablation of androgen receptor in the female adrenal cortex was achieved, with AR ablation confirmed via immunostaining. This provided a suitable model to investigate the role of AR in the female adrenal. Due to AR being X-linked the first generation are mosaic for AR in the adrenal cortex due to random X-chromosome inactivation, this required a second generation to completely ablate AR from steroidogenic adrenocortical cells in the female adrenal. Inclusion of both generations of Ad-ARKOs in the study did however provide the opportunity to investigate the resulting phenotype of partial and complete loss of androgen signaling from the adrenal. Results highlighted that following complete ablation in 2nd generation Ad-ARKO females, significantly less were being born or surviving after birth, although the mechanisms underpinning this is still not clear. These data demonstrate that AR is dispensable in postnatal development of the adrenal cortex and Ad-ARKO females do not show any differences in morphology when compared to littermate controls. Investigation into the effects of AR ablation in the X-zone reveals that partial and compete AR ablation results in X-zone expansion and migration through the cortex, however it is not required for regression during pregnancy. Following ablation of AR the development of spindle cell hyperplasia is observed in the adrenal cortex which is rare in young adults. These results highlight new roles for AR in the female adrenal and suggests that AR protects against the early development of spindle cell hyperplasia.

One of the main issues when carrying out this study was the relatively small number of 2nd generation Ad-ARKO females that were present. Over a long period it was noted that second generation litters frequently had none or very few Cre+ females. To investigate this observation in more depth, a Chi squared test was used (315). This provides the ability to analyze expected litter ratios against observed ratios. This test highlighted that there were significantly fewer second generation Cre+ females being born. The dams from these breeding pairs were AR^{flox} females with no gene ablation so there should not be a maternal or intrauterine issue. The HPA-axis has been shown to be important during foetal development (1), this potentially could be perturbed

following loss of AR. However, continued breeding of this line would be needed to rule out that this result is an artifact and is due to chance.

Initial observations of body weight and adrenal weight during postnatal development, show that the adrenal AR is not required for normal adrenal processes during postnatal development and puberty, which is in contrast to what is observed in Ad-AKRO male littermates. No differences can be detected until adulthood, where a significant increase in adrenal weight can be seen in partial and compete AR ablated female adrenals. Coinciding with this increase in weight in adulthood, large cell clusters can be observed in both generations of female Ad-ARKOs, with a more severe phenotype present in complete AR ablated adrenals. This highlights an important relationship with the amount of AR present and the severity of the cell clusters present in the cortex. Histological analysis revealed the cell clusters in the adrenal cortex were spindle shaped, characteristic of 'type A' cells that make up spindle cell hyperplasia. Spindle cell development has been documented across a number of mouse strains (IQI/jc) (299) and these models have been used in the investigation of the development of adrenocortical tumors (ACTs)(287). These studies highlighted that the development of spindle cell hyperplasia is common in older mice however its expression in young mice is extremely rare. Mouse models describing early development of spindle cell hyperplasia have suggested that they are integral in understanding the development of ACT's, highlighting that an AR ablation model can help understand the contributing factors of ATC development.

As spindle cells can arise naturally in older mice, an analysis was performed that scored presence or absence of spindle cells in histological sections. This demonstrated that 80% of mice that had complete AR ablation in the adrenal cortex had spindle cell hyperplasia, compared to only 20% in littermate controls, suggesting that AR potentially can protect against the development of spindle cell hyperplasia. In addition to the disruption caused by spindle cell development, it could also be observed that the X-zone had expanded and migrated through the cortex. This suggests that female X-zone regression is also controlled by AR, as seen in Ad-ARKO male littermates.

To establish if the presence of spindle cell hyperplasia or X-zone enlargement could be impacting the adrenals stress response and to determine if the disruption to the cortex results in changes to cortical markers, analysis of corticosterone, AKR1B7 and 20 alpha-HSD was performed. Analysis of serum corticosterone revealed no differences in partial or complete ablated female adrenals when compared to controls. This result suggests that there is no impact on the HPA-axis as a result of AR ablation, however, there is variability observed in the serum samples of all cohorts analyzed. This could be in part due to females not being collected in the same time during their estrus cycle. Corticosterone levels have been shown to fluctuate throughout estrus (325), re-sampling during a selected window could provide a more reproducible result and help clarify if there is in fact no changes to corticosterone or impact on stress response. No changes were observed in *Akr1b7* transcript, however, changes in protein localization could be seen. In partial and complete ablated adrenals large portions of the adrenal no longer stained positive for AKR1B7. This change in localization is important for a number of reasons, first it confirms the disruption to the cortex zones supporting the morphology analysis, and additionally AKR1B7 plays an important functional role in the adrenal cortex as well as serving as a zona fasciculata marker. AKR1B7 is responsible for detoxifying isocaproaldehyde generated by steroidogenesis (326). Disruption to AKR1B7 could lead to the buildup of toxins in the adrenal driving further damage to the adrenal cortex. This result is strengthened by the disruption to AKR1B7 observed in Ad-ARKO male littermates, highlighting that androgen signaling, not SF1 (298) could be regulating its expression in the adrenal as it does in the vas deferens (297).

Significant upregulation of 20 alpha-HSD transcript can be observed in partial and complete adrenal Ad-ARKOs, this coincides with X-zone expansion observed morphologically and again through 20 alpha-HSD localization. X-zone cells in the virgin female adrenal cortex typically show concentric organization at the cortex medulla boundary adjacent to the other cortex zones (24, 281). Localization of 20alpha-HSD in Ad-ARKO females shows foetal cells that are dispersed throughout the cortex, a similar phenotype has been previously described in *Prkar1a* adrenal ablation models and suggested that the development and maintenance of foetal cells

(or foetal precursor cells) predisposes these females to manifest a more severe form of primary pigmented nodular adrenocortical disease (PPNAD)(29).

As the adrenal plays an important role in steroidogenesis (280, 318), analysis of steroid marker 3βHSD was performed. Results reveal that spindle cells were not steroidogenic, suggesting that large portions of the outer adrenal cortex taken up by spindle cells is now unable to produce steroids. Spindle cells have also been shown in a number of studies to be a precursor for the development of benign and malignant adrenocortical tumors (ACT) (287, 299, 320). Additionally, spindle cells stained positive for GR. A high percentage of spindle cell tumors are benign and do not cause any further complications, however some of these go on to be malignant and metastatic. The reasons for this is still not clearly understood and early detection of these malignant cells are essential to treatment or identification of adrenocortical tumors. The presence of GR in spindle cells has been shown to highly correlate with ACTs that go on to become malignant carcinomas (320). This suggests loss of AR signaling in the adrenal not only results in spindle cell hyperplasia, but spindle cells that are primed for malignancy.

Examination of spindle cell progression with age reveal, consistent with the literature, that littermate controls develop spindle cell hyperplasia by 12m in 80% of the samples analyzed, and in 100% of Ad-ARKO females. The presence of spindle cell hyperplasia becomes similar to that of littermate controls at 12 months, however, the severity of spindle cell hyperplasia in Ad-ARKOs appears to be more advanced than controls, occupying the majority of the outer cortex rather than being contained to a certain region of the outer cortex, these cells can also be seen protruding down into the ZF. Differences in morphology of the X-zone can also be observed between 12m Ad-ARKOs and 12m littermate controls. The X-zone is known to regress in aged females providing they have not had a pregnancy (34). This can be seen in littermate controls with X-zone shrinking and the influx of blood vessels to this region. This is not observed in the 12m Ad-ARKOs, as the X-zone has expanded and occupies a large portion of the adrenal cortex. These animals also show a decrease in adrenal weight, despite progressive spindle cell and X-zone development. This could potentially be a result of loss of the ZF. Unfortunately, during the aging experiment some mice died

due to age related illnesses, resulting in a sample size of three for the weight and corticosterone measurements. Whilst spare age appropriate tissue was obtained from sample stocks for the morphology analysis, there was no weight or body recordings for these animals. Whilst there was a significant decrease in the adrenal weight and corticosterone in 12m 1st generation Ad-ARKO mice, the power may not be high enough to rule out that these results could have occurred through chance. Whilst interesting, additional animals would have to be analyzed to ensure the results are accurate.

To establish if the X-zone regression during pregnancy is under the control of AR in the female adrenal complete Ad-ARKOs were examined after a pregnancy (parous) and compared to WT BL6 parous female adrenals. These results demonstrate that AR signaling is dispensable during X-zone regression in pregnancy, which is in contrast to what is observed in Ad-ARKO male littermates during puberty. This suggests that its regression during pregnancy is under the control of a different pathway, potentially *Prkar1* as they note the maintenance and progression of the rodent X-zone in parous *Prkar1* knockout females. Despite this, X-zone expansion does occur in 12m Ad-ARKO females, it was hypothesized by Starkey *et al* that the small amounts of circulating testosterone in females is what leads to the eventual regression of the X-zone with age (36) and loss of AR is preventing this from occurring. Despite no changes being observed in X-zone regression in parous Ad-ARKOs, a significant increase in corticosterone was observed. It has been noted that fluctuations of hormones during pregnancy can on occasion result in a psychiatric condition in post-partum women. Development of postpartum depression has been associated with dysregulated HPA-axis and elevated cortisol levels (233, 327). Although more experiments would be needed to investigate this relationship, this data could point to a role for adrenal AR during pregnancy and postpartum stress regulation.

Together this data highlights an important role for AR in the female adrenal, independent of what can be observed in male Ad-ARKOs. Results demonstrate that dysregulation of adrenal AR permits spindle cell hyperplasia development and its loss results in early development of ACTs, additionally, ACTs that express GR previously correlated with malignancy.

Chapter 5: The Cyp11a1-Cre not only permits investigation of the adrenal cortex, but provides a novel model to investigate GR signalling in the hindbrain and its impact on the HPA-axis

5.1 Introduction

The HPA axis is part of the body's neuroendocrine system which regulates responses to internal and external stressors. The HPA axis responds to a stressor by detection in the hippocampus that signals to the hypothalamus to release corticotrophin releasing hormone and arginine-vasopressin (AVP) from the paraventricular nucleus (PVN). Once released into the hypothalamic portal circulation CRH induces the production of adrenocorticotrophic hormone (ACTH) by the corticotrophs of the anterior pituitary. ACTH in turn stimulates the adrenal cortex to release the glucocorticoids cortisol (in humans) or corticosterone (in rodents) (109, 232, 316, 317). This activation results in low affinity for food, low sex drive, increased blood flow to muscle, increased locomotor activity and raised blood sugars which prime the body to respond to a stress event (232). In normal conditions these bursts of activity last for only a few minutes at a time, however, prolonged stress is thought to overstimulate the HPA axis causing hypersecretion of CRH and can ultimately, if left untreated, lead to the dysregulation of the HPA axis (233). Glucocorticoids and glucocorticoid receptor (GR) are essential in regulating this process. GR is a nuclear steroid hormone receptor. Binding of glucocorticoids to GR permits the repression or induction of transcription of target genes, this occurs through binding to DNA response elements or through interactions with other transcription factors. GR signalling has been shown to be important in a wide array of biological systems, such as immunosuppression (328), metabolism (329), inflammatory response (330), cell proliferation (331), and animal behaviour (332). Due to the numerous biological processes controlled by GR signalling and the widespread expression of GR, understanding this pathway and its interactions is essential. The potential for GR signalling being able to directly target the adrenal was demonstrated in the rodent adrenal in the 1970s, describing adrenal atrophy in hypophysectomised rats treated with glucocorticoids which showed adrenal cortex shrinkage and impaired adrenal function (333). More recent studies have identified the

presence of GR in the human adrenal through foetal development and its presence in the development of diseases such as primary pigmented nodular adrenocortical disease and in adrenocortical tumours (320, 334-336).

GR signalling interactions are complex and results obtained in the previous two chapters have highlighted a potential relationship between AR and GR in the adrenal cortex of the mouse adrenal. Interactions between AR and GR have been noted in the literature in various tissues throughout the body, some of these examples include the negative regulation of GR by AR in prostate cancers, and this study demonstrated an inverse correlation between AR activity and GR protein expression during prostate cancer progression (203). However, the converse relationship has been demonstrated in human adipocytes where glucocorticoid signalling inhibited AR (337). In addition to these studies, it has also been demonstrated that AR and GR have a high degree of sequence homology, especially in regards to their DNA-binding domains. They both bind to a DNA site termed a hormone response element, this is thought to permit extensive cross talk between the two receptors (205). Again, this relationship was demonstrated in prostate cancer treatment, where cancer progression and metastasis was achieved through GR targeting and upregulating genes controlled by AR and thus negating enzalutamide treatment (338). Despite this relationship being highlighted in various tissues, the relationship and potential crosstalk between AR and GR in the adrenal cortex has yet to be defined. For these reasons, the same *Cyp11a1*-Cre utilised in chapters three and four was again used to try and target GR in the adrenal cortex, to generate a novel model to investigate GR signalling in the adrenal, investigate its impact on the HPA-axis, and investigate the impact of androgen signalling in the adrenal cortex as a result of GR ablation.

5.1.1: Preliminary data

5.1.1.1Cyp11a1-Cre fails to target GR expressing cells in the adrenal cortex

The initial aim was to target GR expressing cells in the adrenal cortex. However, double immunofluorescence analysis for YFP and GR in *Cyp11a1*-YFP reporter mice shows that the Cre does not target GR expressing cell types in the adrenal (Fig. 5-1A), and immunohistochemistry confirms no GR ablation in the adrenal cortex of adrenal GR knockout (Ad-GRKO) mice (Fig. 5-1B). GR was found to be localised in the small pockets between the cell types expressing the Cre. Unfortunately this highlights that the *Cyp11a1*-Cre was unsuitable for the intended purpose and was not able to target the adrenal as originally intended. The adrenal cortex receives signals from multiple organs, two most notably are the brain and the pituitary in the regulation of the HPA-axis. It is essential that signalling from both these organs do not become perturbed, if they do, this can lead to a number of disorders that can over or under stimulate the adrenal cortex ultimately resulting in adrenal cortex damage and inappropriate action of the adrenal.

Despite not being able to target the required cell types in the adrenal, the *Cyp11a1*-Cre does have the potential to target the hindbrain (253). For these reasons, interrogation of the genomic hindbrain DNA was performed. Investigation of hindbrain genomic DNA shows recombination of GR, highlighting that the Cre has targeted the brain (Fig. 5-1C). These results demonstrate that any subsequent phenotype arising in these mice results from ablating GR specifically from the hindbrain. Various studies have demonstrated that mutations of GR in the brain can disrupt the adrenal cortex through a dysregulated HPA-axis and can result in psychiatric conditions. Identifying new and appropriate models to investigate stress and their impact on the adrenal are extremely useful (178). Previous studies have highlighted the role of GR in the brain and its control of stress and cognitive function (234). For example, it has been shown that glucocorticoids acting through GR in the hindbrain can enhance the arterial pressure in response to acute restraint stress (235) suggesting that GR is required for an appropriate stress response in healthy individuals. Persuasive evidence points to dysfunctional GR in HPA axis dysregulation, which have been successfully resolved upon antidepressant treatment (232). These claims are backed by transgenic studies

that use antisense RNA complimentary to mRNA to knock down GR in the brain and these mice display characteristics of depression in addition to altered behaviour (236). For these reasons, the initial aims were revised to address the role of hindbrain GR and its impact on the adrenal cortex (5.1.2-3).

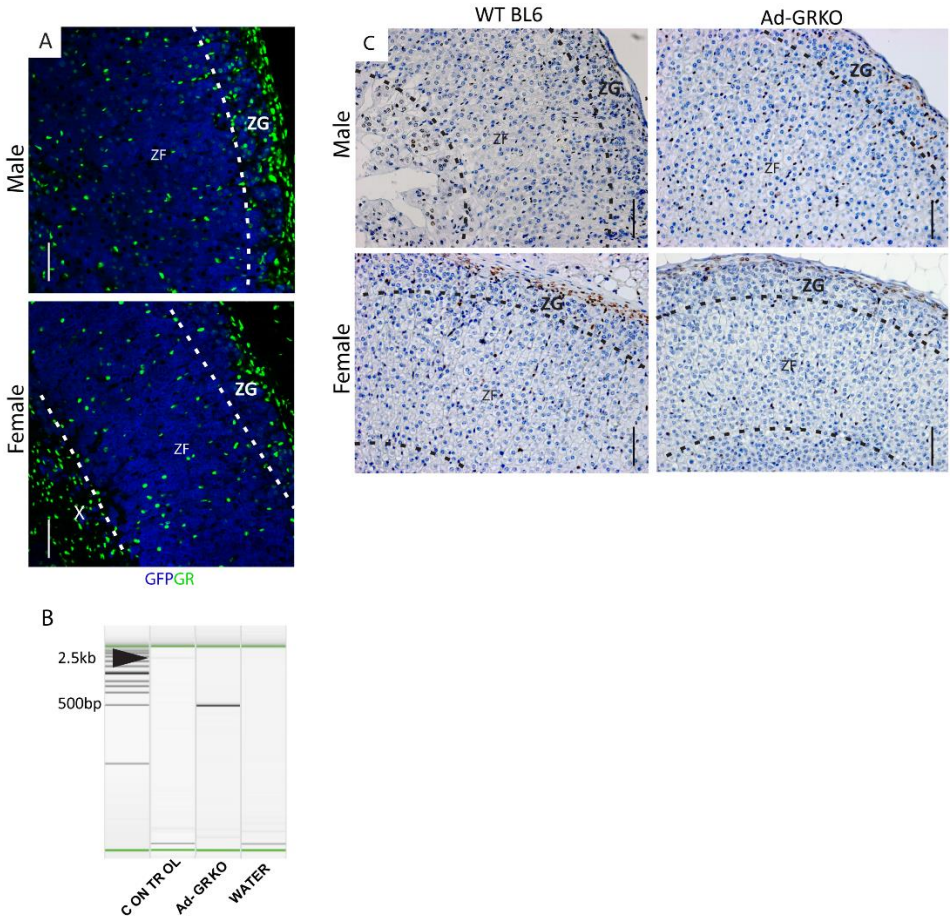


Figure 5-1. Cyp11a1-Cre does not target GR expressing cells in the adrenal cortex. ***(A)*** *Immunohistochemical localisation of GFP (blue) and GR (green) in the adrenal of Cyp11a1$^{+/GC}$: R26-EYFP mice show that despite the Cre expression in the adrenal cortex, it does not target GR positive cells.* ***(B)*** *PCR interrogation of genomic PCR confirms recombination of floxed GR in the hindbrain. Control sample demonstrated by 2.5kb fragment, recombined GR demonstrated by 500bp fragment.* **(C)** *Immunohistochemical localisation of GR in the adrenal cortex of Ad-GRKO shows that GR has not been targeted in the adrenal cortex and show no differences in localisation when compared with littermate controls. Scale bars 50µm. Abbreviations; ZG=zona glomerulosa, ZF= zona fasciculata.*

5.1.2 Initial aims

- To generate specific GR ablation in the adrenal cortex through the use of *Cyp11a1*-Cre
- To establish the impact of GR ablation in the adrenal cortex and HPA axis
- To determine if there is any impact on androgen signalling

5.1.3 Revised aims

- To establish the impact of GR ablation in the hindbrain on the adrenal cortex
- To determine if hindbrain GR ablation leads to abnormal behaviour
- To define any potential impacts on androgen signalling

5.2 Results

5.2.1 Animals used in this study

A number of mouse lines were ultimately investigated as part of this study. For clarity, genotypes and abbreviations are detailed below in table 5-1. Breeding of these animals is detailed in materials and methods section 2.1.3.4.

Genotype	**Abbreviation**
Cre- GRf^{lox} Homozygous	'littermate control' (LMC)
Cre- GRf^{lox} Heterozygous	'Het littermate control' (Het LMC)
Cre+ GRf^{lox} Homozygous	'Hindbrain GR knockout' (HB-GRKO)
Cre+ GRf^{lox} Heterozygous	'Het hindbrain GR knockout' (Het HB-GRKO)
c57bl/6J wildtype	'WT Bl6'
GR floxed	'GR^{flox}'

Table 5-1. Genotypes and abbreviations.

5.2.2 HB-GRKO mice show stressed behaviours via excessive barbering and digging

As disruption to GR is known to play a role in stress and anxiety (230, 232, 339), 40 cages consisting of between 4-6 male or female mice of mixed genotypes were first observed for signs of behaviour consistent with stress. Monitoring of cage behaviour via video recording prior to collection revealed that WT BL6 mice explored the cage with multiple rearing attempts made (normal behaviour associated with the explorative nature of mice) (340). By contrast, HB-GRKO mice spent little time exploring and focused on repetitive digging in the same spot, behaviour characteristic of a stressed state (Fig. 5-2A)(341)

Barbering in mice is a normal social behaviour although excessive or abnormal barbering has been associated with stress and anxiety (263, 264). Both male and female HB-GRKO mice display excessive barbering resulting in hair loss from large portions of the face, back and stomach (Fig. 5-3A). HB-GRKO mice are also observed barbering LMCs, and this suggested that HB-GRKO mice were the cause of the excess cage barbering. In support of this, individually housed HB-GRKO males self-barbered, frequently resulting in large portions of hair removal. (Fig. 5-3B). However, this was not observed in individually housed LMC males (Fig. 5-2C). Mice were assigned a 'barbering score' (outlined in section 2.3.3.7) based on a previously determined scale (263, 264). WT BL6 mice were recorded to have a barbering score between 0-1 whereas HB-GRKO mice were given a barbering score of between 3 and 5, which is above the accepted limit for normal husbandry barbering. Cages containing a higher number of HB-GRKO animals demonstrated some of the most severe barbering, all falling within 4-5 on the barbering scale (Fig. 5-3D). Interestingly, stress behaviour and severe hair loss through barbering does not present until at least d90. Because the apparent interaction between HB-GRKO mice and their littermates leading to observable phenotypes in all genotypes, which may act as a confounder, an external C57BL6 cohort was also included in the analysis to act as a further control. These mice were bred and held in the same rack as the HB-GRKO colony but had no direct contact with HB-GRKO mice, and displayed no evidence of excessive barbering. Together this data suggests that loss of GR signalling in the hindbrain results in increased stress-related behaviour.

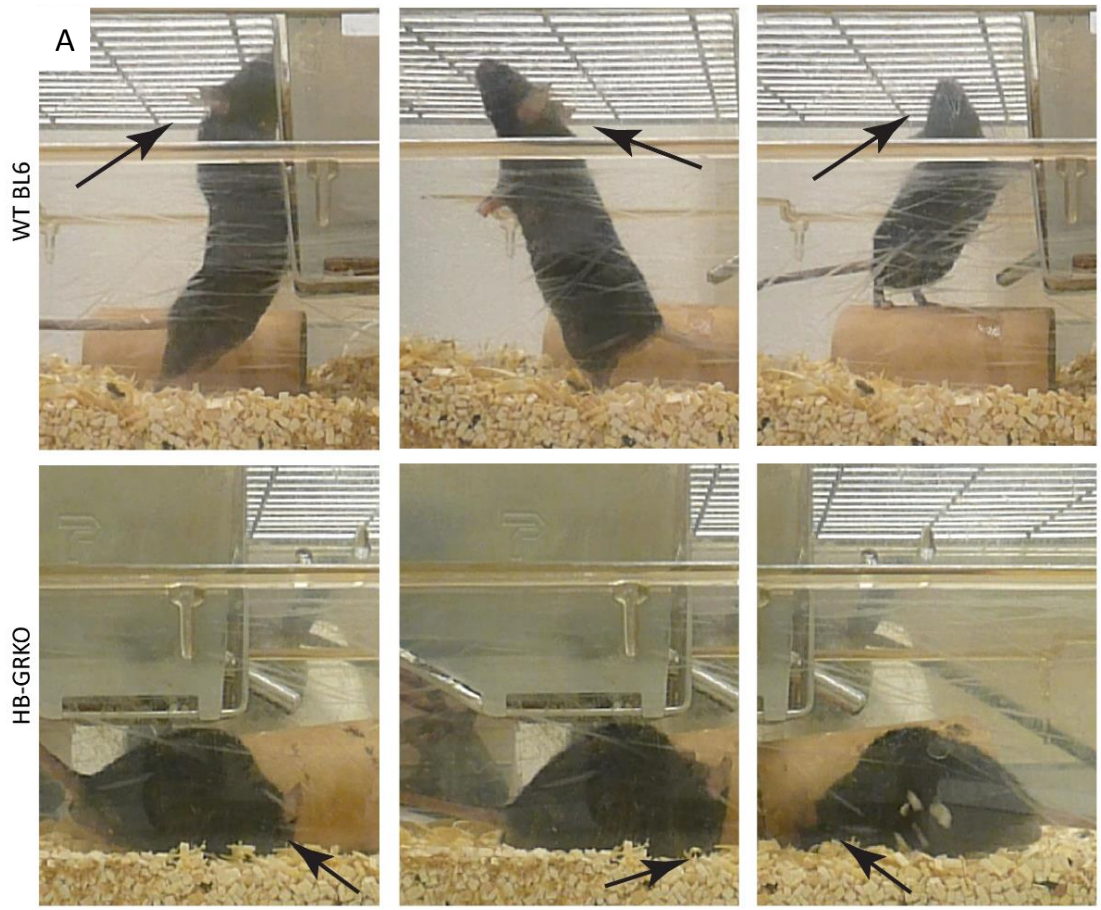


Figure 5 -2. HB-GRKO mice display behaviour consistent cage stress. (A) *Observations of cage behaviour over a 5 minute period. Control animals can be seen exploring that cage with multiple rearing observed. HB-GRKO mice did little cage exploration and displayed obsessive behaviour via excessive and compulsive digging. Rearing and digging behaviour denoted by arrows.*

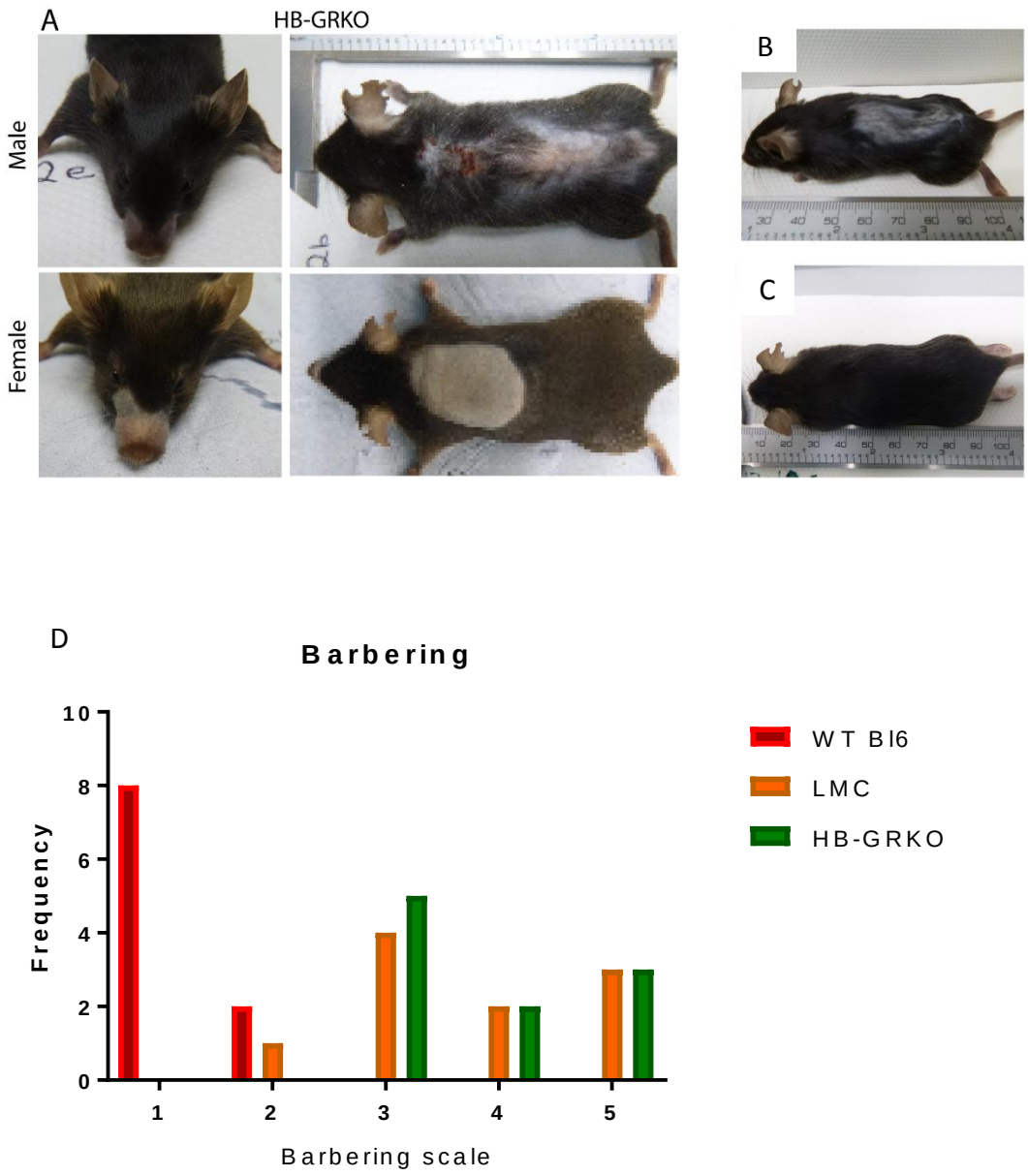


Figure 5-3. HB-GRKO mice display moderate to severe barbering. ***(A)*** *Whole body examination shows excessive barbering to face and back in male and females, often resulting in broken skin and complete removal of whiskers.* ***(B)*** *Individually caged HB-GRKO male showing excessive barbering and broken skin.* ***(C)*** *Individually caged LMC male displaying no hair loss or barbering.* ***(D)*** *Histograph detailing distribution of genotypes and barbering severity. Scale bars 1cm.*

5.2.3 CT/PET imaging confirms kyphosis in HB-GRKO mice

The excessive barbering observed of LMCs suggests that they could be affected by HB-GRKO mice, for these reasons an external WT Bl6 male and female cohort was included in body weight and adrenal weight measurements. Body weight is significantly increased in HB-GRKO males compared to male WT BL6 mice (Fig. 5-4A), whilst in contrast, a significant decrease in body weight is observed in female LMCs and HB-GRKO mice compared to female WT BL6 mice (Fig. 5-4B). Analysis of body weights in heterozygous males show no differences in weight compared to WT bL6 males (Fig. 5-4C), however heterozygous females show the same significant decrease in body weight when compared to WT bL6 females (Fig. 5-4D). During collection and weighing it could be seen that HB-GRKO mice have excessive spinal curvature in both males and females (Fig. 5-4E). To investigate this further, micro-CT scans were taken, WT BL6 mice were not included in this part of the analysis as LMCs did not show increased spinal curvature. These results reveal an increase in spinal curvature in HB-GRKO male and female mice compared to LMC male and females (Fig. 5-5A). This accentuated spinal curvature was also observed in Het HB-GRKO males and females compared to Het LMCs (Fig. 5-5B). Closer inspection of the vertebrae was used to establish whether this curvature is a result of vertebral fusing, however, results show no fusing of the vertebrae in any group analysed (Fig. 5-6A). The absence of vertebral fusing in these mice suggests that the curvature of the spine is not due to a disorder in development. As used in standard orthopaedic practice, the inward angle of the spine was measured to quantify the extent of the curvature observed in HB-GRKO and Het HB-GRKO mice (342). These measurements confirm increased spinal curvature in HB-GRKO mice compared to LMCs, highlighting kyphosis development (Fig. 5-6B-E).

Elevated levels of serum corticosterone have been linked to the development of kyphosis in Cushing's patients (300), so corticosterone was measured in these mice. This revealed significantly elevated levels in HB-GRKO, Het HB-GRKO and LMC males compared to WT BL6 mice (Fig. 5-7A), this increase was not observed in any female cohort compared to WT BL6 females (Fig. 5-7B)

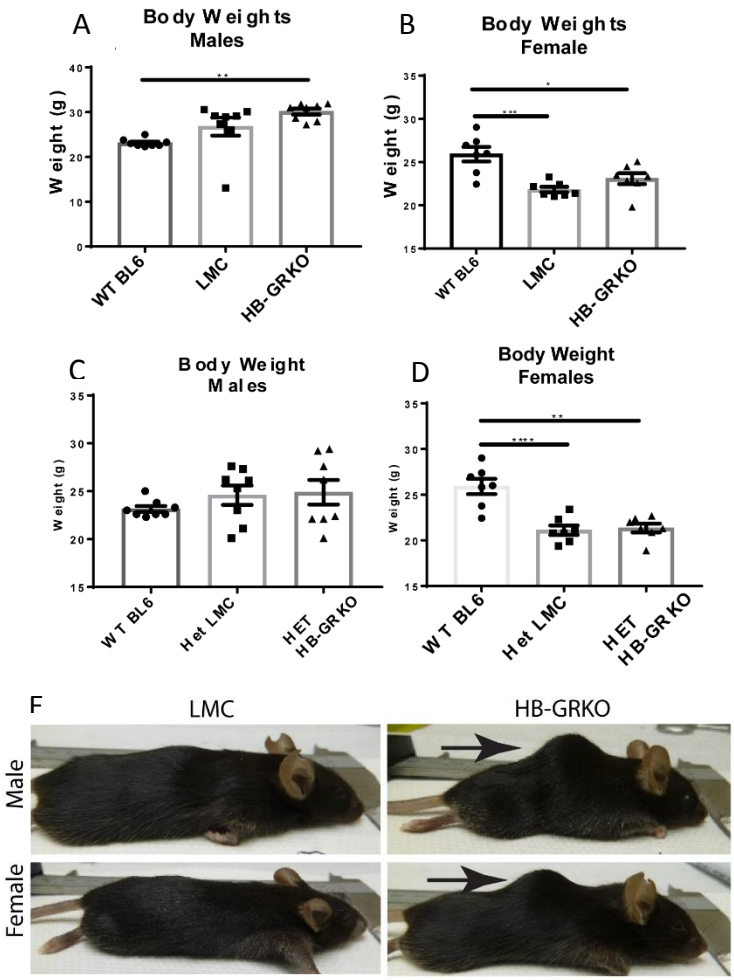


Figure 5-4. Excessive spinal curvature can be seen in HB-GRKO mice.

*(A) Male body weights compared to an external WT BL6 cohort reveals a significant increase in HB-GRKO mice (one-way ANOVA; n=7-8, **p<0.001, Tukeys post-hoc analysis, error bars SEM). (B) Female body weights compared to an external C57BL6 control show a significant decrease in both in HB-GRKO LMC and HB-GRKO mice (one-way ANOVA; n=8, ***p<0.0001, *p<0.05, Tukeys post-hoc analysis, error bars SEM). (C) Male body weights of Het mice compared to an external WT BL6 cohort reveals no difference between the cohorts. There is no difference in weights in HB-GRKO LMC compared to BL6 controls. (D) Female body weights of Het mice compared to an external WT BL6 cohort show a significant decrease in both in HB-GRKO LMC and HB-GRKO mice (one-way ANOVA; n=8, ****p<0.0001, **p<0.001 Tukeys post-hoc analysis, error bars SEM). (E) Whole body images demonstrating exaggerated spinal curvature in HB-GRKO mice.*

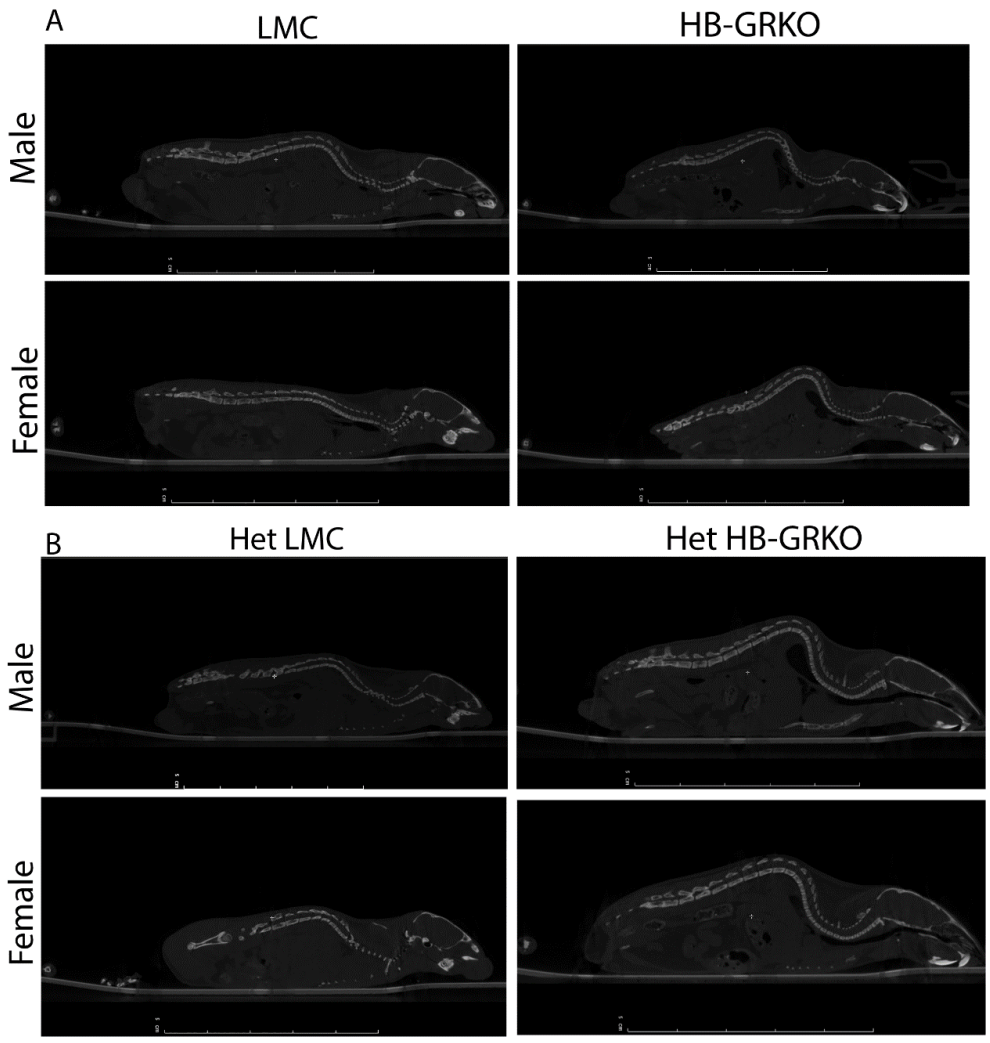


Figure 5-5. Spinal curvature examination via CT/PET analysis. **(A)** *CT scans show that the spine in HB-GRKO mice have an increase in spinal curvature and show spinal collapse in HB-GRKO mice compared to littermate controls.* ***(B)*** *CT scans show that the spine in Het HB-GRKO mice have an increase in spinal curvature and show spinal collapse in HB-GRKO mice compared to littermate controls. Scale bar 5cm.*

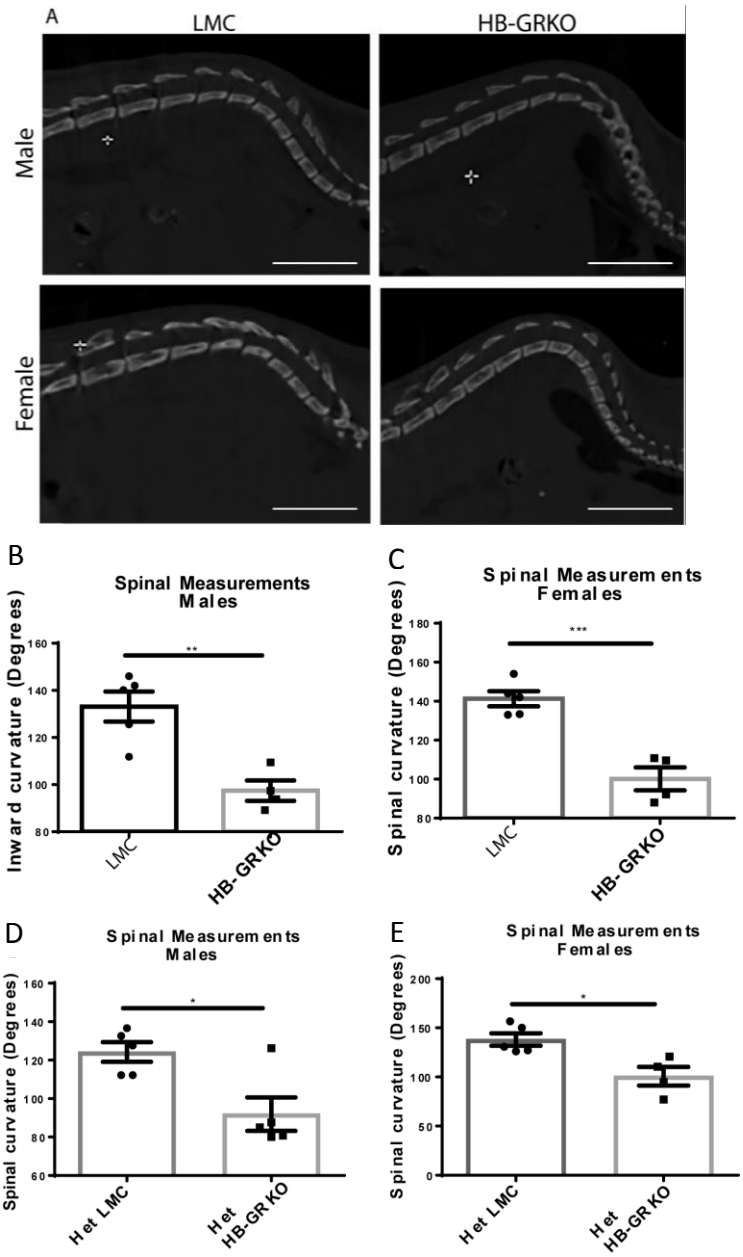


Figure 5-6. Excessive spinal curvature confirmed through Cobb analysis. ***(A)*** *Closer investigation of the spine shows no fusing of vertebrae in HB-GRKO.* ***(B, C)*** *Interrogation of the anterior angle of the spine shows a significant decrease in HB-GRKO mice when compared to littermate controls. (Unpaired Student T-test;**p<0.001, ***p<0.0001. Error bars SEM).* ***(D, E)*** *Interrogation of the anterior angle of the spine shows a significant decrease in Het HB-GRKO mice when compared to littermate controls. (Unpaired Student T-test;*p<0.05, *p<0.05. Error bars SEM).*

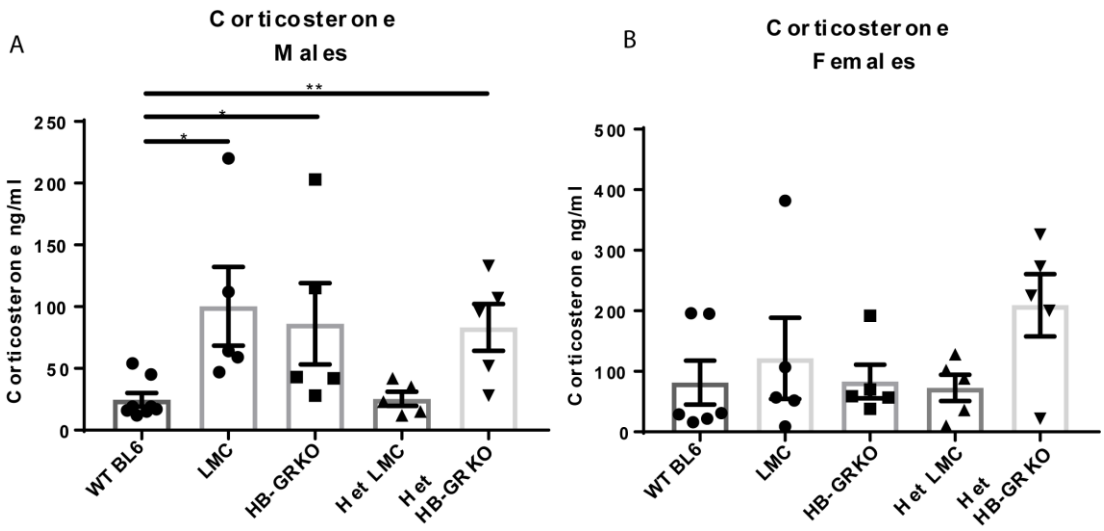


Figure 5-7. Increased circulating corticosterone observed in male HB-GRKO mice. (A) *Circulating corticosterone levels are elevated in both male HB-GRKO LMC and HB-GRKO mice compared to WT BL6 mice (One-way ANOVA; n=5-8, *p<0.05, *p<0.05, Tukeys post-hoc analysis, error bars SEM). This increase is also observed in Het HB-GRKO mice (One-way ANOVA; n=5-8, **p<0.001, Tukeys post-hoc analysis, error bars SEM), but not Het LMCs* ***(B)*** *Circulating corticosterone levels do not change in any female cohort compared to WT BL6 controls.*

5.2.4 HB-GRKO leads to severe adrenal cortex disruption

Due to the apparent stress behaviours displayed by HB-GRKO mice and the relationship between the adrenal gland and stress response (109) (e.g. raised corticosterone), we examined the adrenal glands to determine whether there are any impacts on their morphology and function. Interrogation of adrenal weights revealed a significant increase in HB-GRKO and LMC male mice compared to WT BL6 males. The same changes in adrenal weight can also be seen in Het LM and Het HB-GRKO males (Fig. 5-8A). No changes in adrenal weight were observed in any female cohort compared to an external WT BL6 control (Fig. 5-8B).

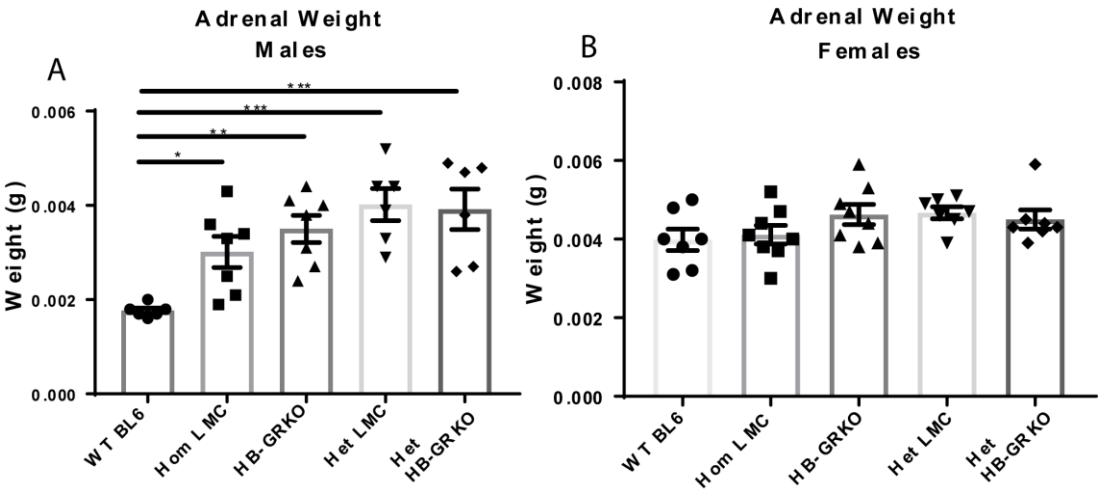


Figure 5-8. HB-GRKO LMC and HB-GRKO present with severely disrupted adrenals. ***(A)*** *Analysis of male adrenal weight revealed an increase in weight in all cohorts compared to WT BL6 controls (One-way ANOVA; n=6-8,*p<0.05, **p<0.001, ***p<0.0001, ***p<0.0001, Tukeys post-hoc analysis, error bars SEM).* ***(B)*** *Analysis of female adrenal weight revealed no differences between any of the cohorts compared to WT BL6 controls.*

Histological analysis of the adrenal in male HB-GRKO mice revealed severe disruption to the cortex with disorganised cortical zones. Interestingly, examination of male LMCs revealed a similar level of disruption to their adrenal cortex. However male WT BL6 mice have no such disruption to the adrenal, suggesting that the impact on LMC adrenals is also likely related to their being housed with HB-GRKO mice (Fig. 5-9A).

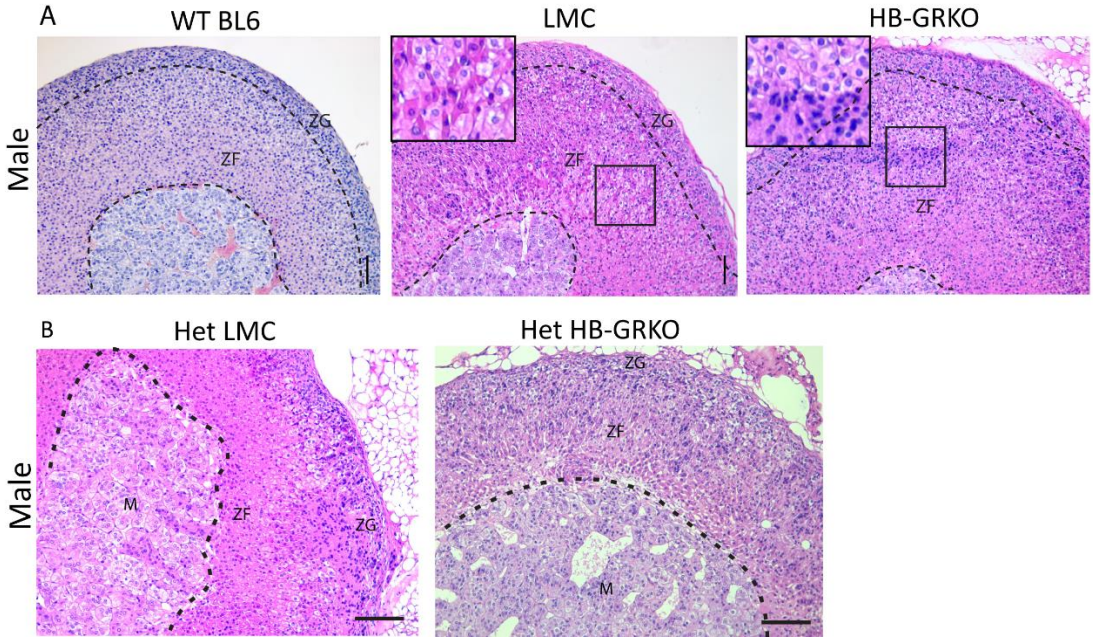


Figure 5-9 LMCs and HB-GRKO males present with severely disrupted adrenals. ***(A)*** *Morphology analysis of male HB-GRKO LMC and HB-GRKO adrenals both display major disruption to the entire cortex. Due to the disruption observed in littermate controls, an external BL6 cohort was included in the analysis. Enlarged images of regions of interest denoted by a box. (B) Morphology analysis of male Het LMC and Het HB-GRKO adrenals again display major disruption to the entire cortex. N=5. Scale bars 100µm. Abbreviations; ZG= zona glomerulosa, ZF= zona fasciculata, X=X-zone, M=medulla.*

The adrenals of female HB-GRKO and LMCs mice show a similar disruption to the cortex as seen in the males, with the addition of enlargement of the X-zone, which occupies a large portion of the cortex compared to the X-zone in female WT BL6 mice which is maintained in a compact zone around the medulla (Fig. 5-10A). The same adrenal cortex disruption can also be seen in Het littermates (Fig. 5-10B), further supporting that littermates are being effected by HB-GRKO mice.

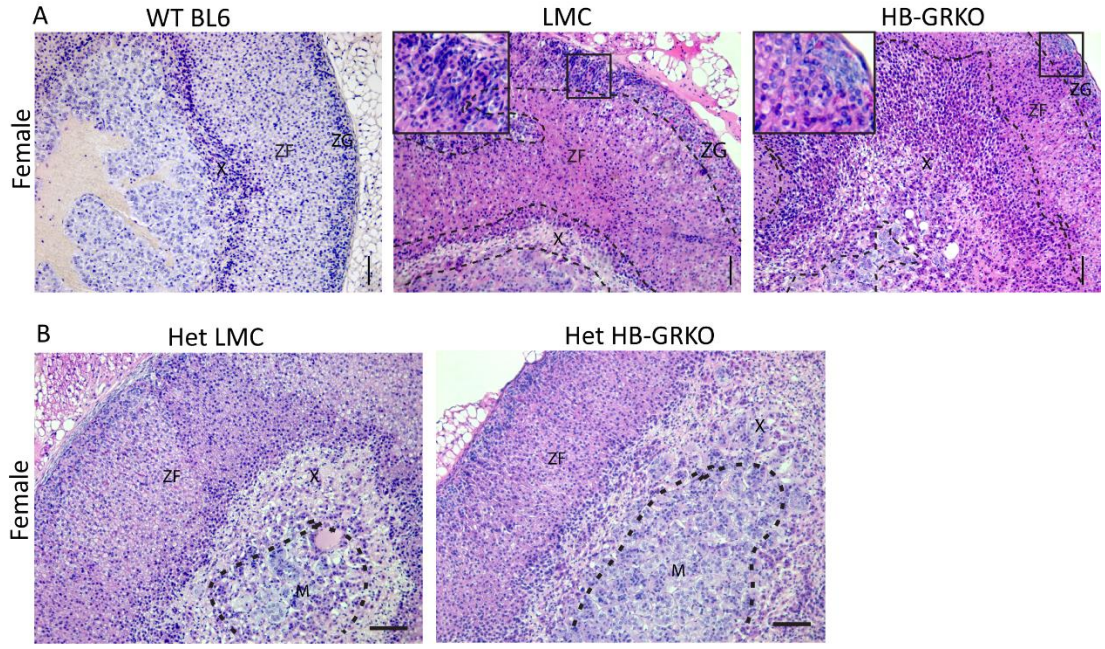


Figure 5-10 LMCs and HB-GRKO females present with severely disrupted adrenals. ***(A)*** *Morphology analysis of female HB-GRKO LMC and HB-GRKO adrenals both display major disruption to the entire cortex. Due to the disruption observed in littermate controls, an external WT BL6 cohort was included in the analysis. Enlarged images of regions of interest denoted by a box.* ***(B)*** *Morphology analysis of female Het LMC and Het HB-GRKO adrenals again display major disruption to the entire cortex and X-zone expansion compared to WT BL6 females. N=5. Scale bars 100µm. Abbreviations; ZG= zona glomerulosa, ZF= zona fasciculata, X=X-zone, M=medulla.*

Due to all genotypes in the cage presenting with a stress phenotype, to rule out that this could be caused by the insertion of the *loxP* sites into exon 3, GR floxed male adrenals were examined and showed adrenal morphology was normal in these animals (Fig. 5-11A).

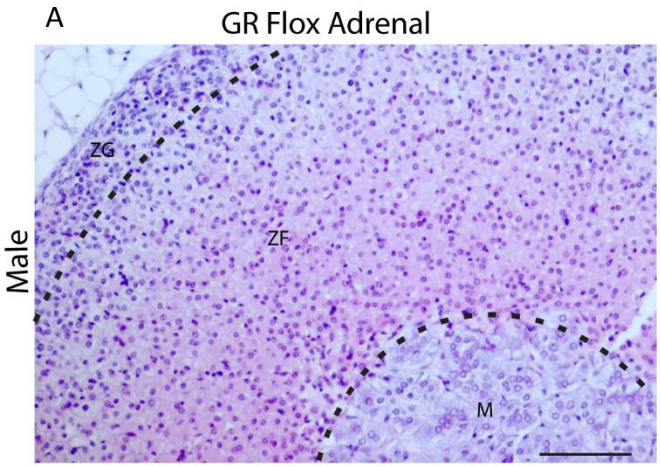


Figure 5-11. Insertion of loxP sites does not result in disruption to adrenal morphology. (A) *Morphology analysis of GR flox adrenals show no disruption to the adrenal cortex. N=4. Scale bars 100µm. Abbreviations; ZG= zona glomerulosa, ZF= zona fasciculata, M=medulla.*

5.2.5 Loss of HB-GRKO leads to disruption to cortical markers 20 alpha-HSD and AKR1B7

Analysis of X-zone marker 20-α-hydroxysteroid dehydrogenase (20 alpha-HSD) (281) localization reveals 20 alpha-HSD positive cells in LMCs and HB-GRKO males, compared to WT BL6 males which normally do not express this marker in adulthood. Fitting with the morphology analysis, Het LMCs and Het HB-GRKO mice show the same disruption to 20 alpha-HSD in the adrenal cortex (Fig 5-12 A, B). 20 alpha-HSD localization analysis in LMCs and HB-GRKO females revealed dispersed positive cells throughout the cortex compared to the compact X-zone observed in WT BL6 females, this disruption again could also be observed in Het LMCs and Het HB-GRKO mice (Fig. 5-13 A, B).

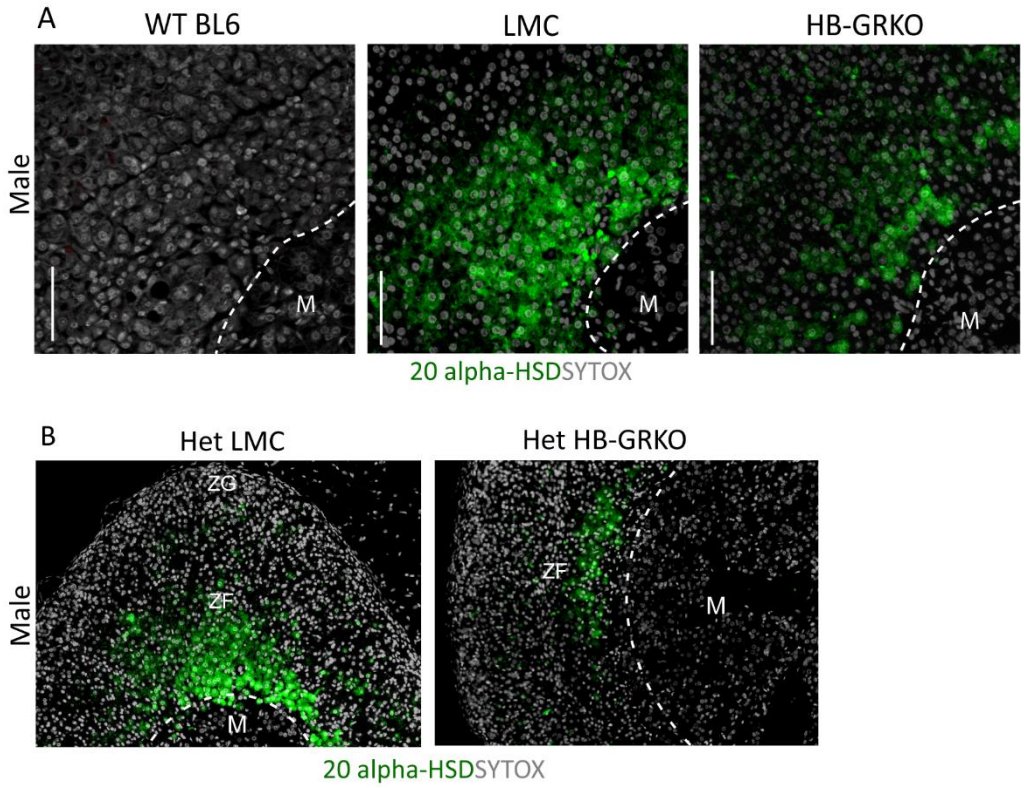


Figure 5-12. Male LMCs and HB-GRKO males show the presence of foetal cells in the adult adrenal cortex. ***(A)*** *Immunohistochemical localisation of 20 alpha-HSD revealed the presence of foetal X-zone cells in both male LMCs and HB-GRKO mice compared to WT BL6 mice in which no expression is normally observed.* ***(B)*** *Immunohistochemical localisation of 20 alpha-HSD revealed the presence of foetal X-zone cells in both male Het LMC and Het HB-GRKO mice also. Green; 20alpha-HSD, Grey; sytox counterstain. N=5. Scale bars 100µm. Abbreviations; ZG= zona glomerulosa, ZF= zona fasciculata, X=X-zone, M=medulla.*

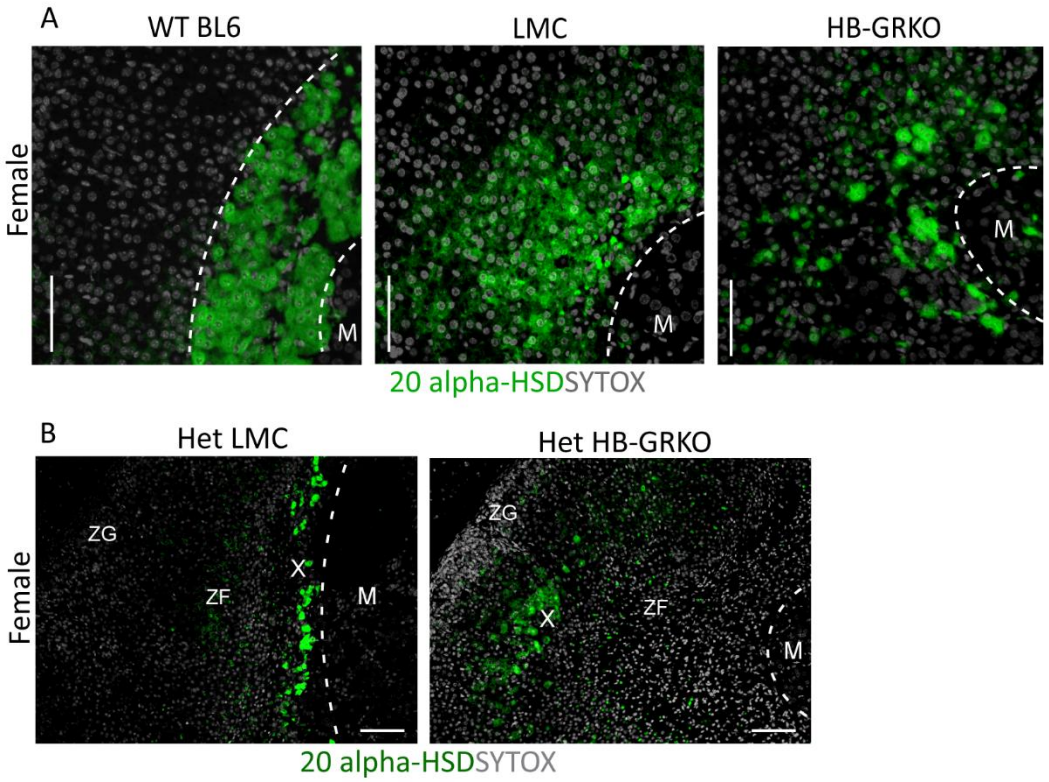


Figure 5-13. LMCs and HB-GRKO females have disrupted and dispersed X-zones. ***(A)*** *Immunohistochemical localisation of 20 alpha-HSD revealed the presence of foetal X-zone cells in both female LMCs and HB-GRKO mice compared to WT BL6 mice in which no expression is normally observed.* ***(B)*** *Immunohistochemical localisation of 20 alpha-HSD revealed the presence of foetal X-zone cells in both female Het LMC and Het HB-GRKO mice also. Green; 20alpha-HSD, Grey; sytox counterstain. N=5. Scale bars 100µm. Abbreviations; ZG= zona glomerulosa, ZF= zona fasciculata, X=X-zone, M=medulla.*

Localization of AKR1B7 a well described zona fasciculata marker (297), showed disruption to both male and female LMCs and HB-GRKO, compared to WT BL6 which normally stains the whole ZF. LMCs display partial loss of AKR1B7 positive cells with HB-GRKO mice showing even fewer positive cells (Fig. 5-14A). Again, Het LMCs and Het HB-GRKO males show the same disruption to AKR1B7 in the adrenal cortex (Fig 5-14B). The same phenotype is observed in females (Fig 5-15 A, B).

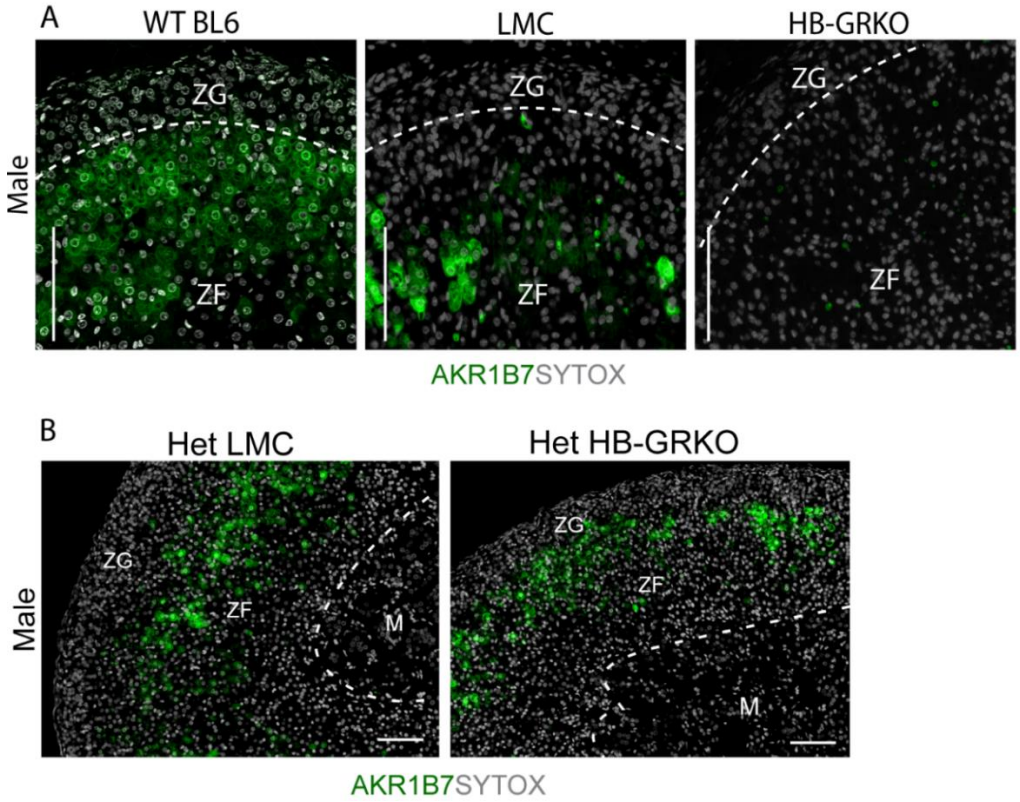


Figure 5-14. HB-GRKO LMC and HB-GRKO males have disruption to AKR1B7. (A) *Immunohistochemical localisation of AKR1B7 revealed disruption in both male LMCs and HB-GRKO mice compared to WT BL6 controls, with fewer AKR1B7 positive cells being observed.* ***(B)*** *Immunohistochemical localisation of AKR1B7 revealed disruption in both male Het LMC and Het HB-GRKO mice, with fewer AKR1B7 positive cells being observed. Green; AKR1B7, Grey; sytox counterstain. N=5. Scale bars 100µm. Abbreviations; ZG= zona glomerulosa, ZF= zona fasciculata, X=X-zone, M=medulla.*

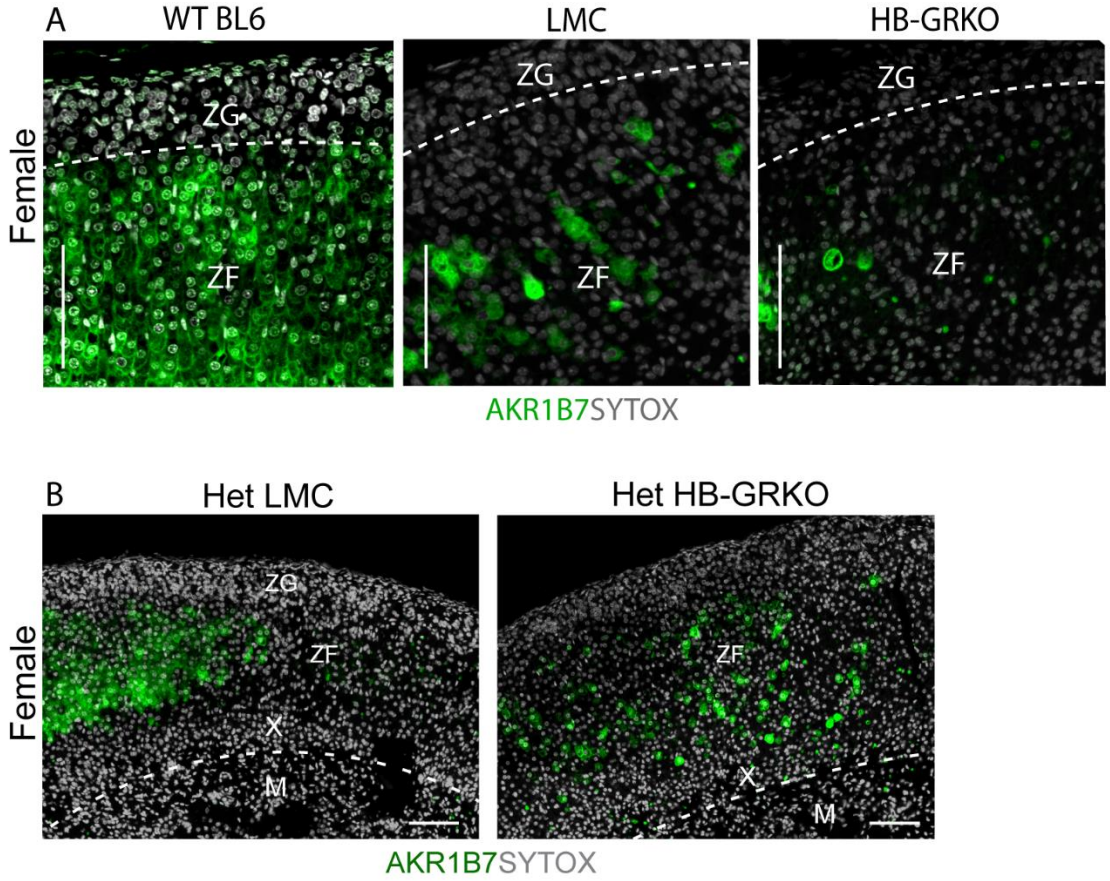


Figure 5-15. HB-GRKO LMC and HB-GRKO females have disruption to AKR1B7. ***(A)*** *Immunohistochemical localisation of AKR1B7 revealed disruption in both female LMCs and HB-GRKO mice compared to WT BL6 controls, with fewer AKR1B7 positive cells being observed.* ***(B)*** *Immunohistochemical localisation of AKR1B7 revealed disruption in both female Het LMC and Het HB-GRKO mice, with fewer AKR1B7 positive cells being observed. Green; AKR1B7, Grey; sytox counterstain. N=5. Scale bars 100µm. Abbreviations; ZG= zona glomerulosa, ZF= zona fasciculata, X=X-zone, M=medulla.*

5.2.6 No changes in circulating androgens or AR can be detected in HB-GRKO mice

Due to the presence of foetal cells in the adrenal cortex in male LMCs and HB-GRKO mice along with the disruption to AKR1B7, these mice displayed a similar phenotype to that observed in Ad-ARKO mice in chapter 3. In addition to this, GR has been shown to be significantly up regulated in mice that are castrated (section 3.2.10). Circulating androgens have also been noted in playing a role in stress response. Mice treated with testosterone were shown to spend more time in open maze tests and increased exploratory behaviour (332, 343). For these reasons circulating testosterone and AR immunolocalisation were analysed to determine if there was any change to AR signalling in the adrenal cortex. Circulating testosterone is elevated in Het HB-GRKO and HB-GRKO males compare to LMCs, however, this increase is not significant (Fig 5-16A). Immunostaining of androgen receptor also showed no changes in localisation in the adrenal cortex (Fig 5-16B).

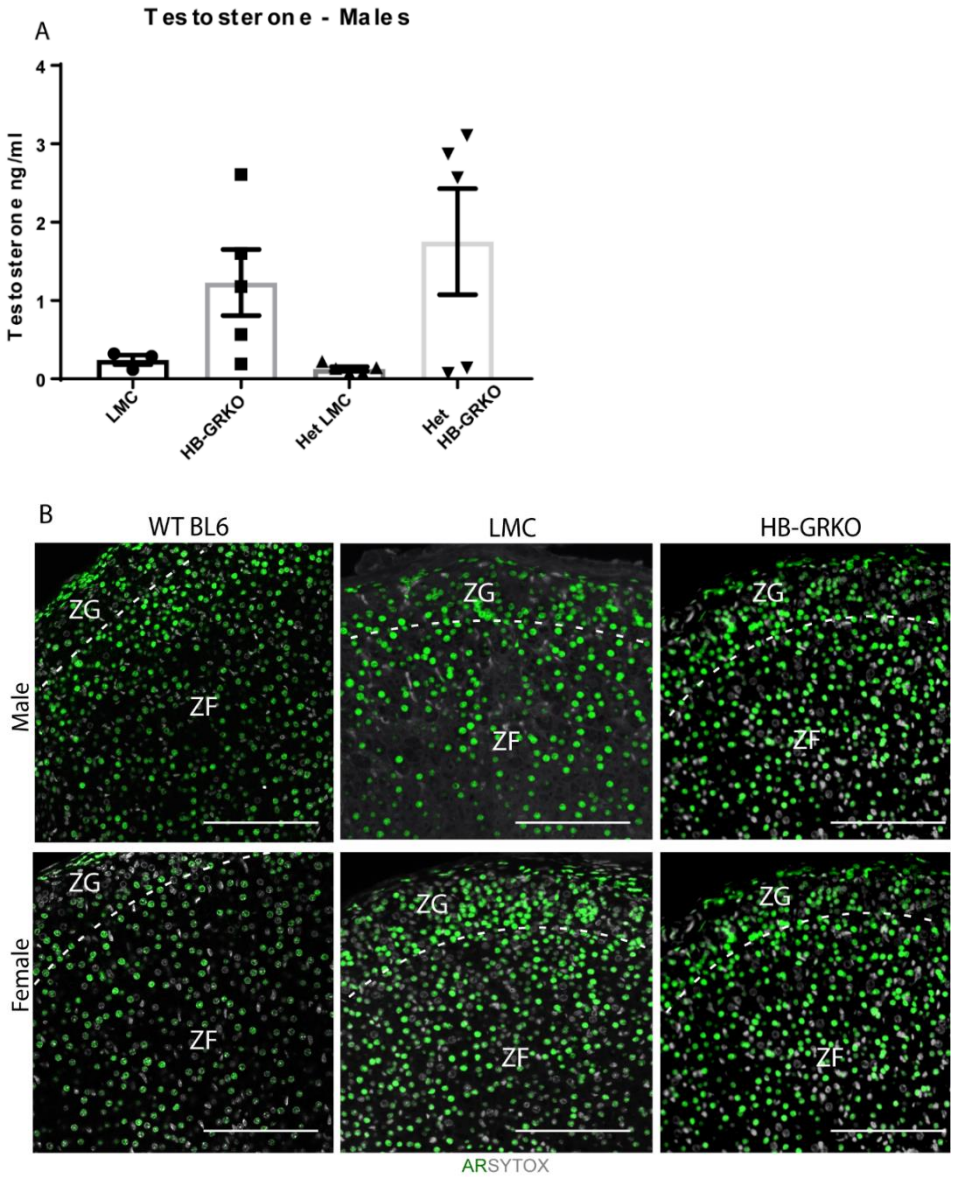


Figure 5-16. No changes in circulating testosterone or AR detected. ***(A)*** *Circulating testosterone in male LMC and Het LMC and Het HB-GRKO and HB-GRKO mice. No significant difference was detected in any cohort analysed however there does appear to be increased circulating testosterone in HB-GRKO and Het HB-GRKO mice. N=5.* ***(B)*** *Immunostaining for AR revealed no differences in any cohort analysed. Green; androgen receptor, Grey; sytox counterstain. N=5. Scale bars 100µm. Abbreviations; ZG= zona glomerulosa, ZF= zona fasciculata, X=X-zone, M=medulla.*

5.2.7 HB-GRKO mice show cell death throughout the adrenal cortex

To establish whether disruption to the adrenal cortex leads to changes in cell clearance, the apoptosis marker cleaved caspase 3 was examined. Normal apoptosis usually occurs at the cortex-medulla boundary however, cleaved caspase 3 localization in male HB-GRKO mice revealed many caspase-positive cells throughout the whole adrenal cortex indicating a large amount of abnormal cell death (Fig. 5-17A). Interrogation of cleaved caspase staining in Het LMCs and Het HB-GRKO males showed caspase positive cells throughout the cortex, however this was not as severe as HB-GRKO males (Fig. 5-17B). Cleaved caspase 3 localization in female HB-GRKO mice again revealed many caspase-positive cells throughout the whole adrenal cortex (Fig. 5-18A) and many positive cells throughout the X-zone. Unlike Het males, Het LMC and Het HB-GRKO females showed similar amounts of cell death throughout the cortex (Fig. 5-18B).

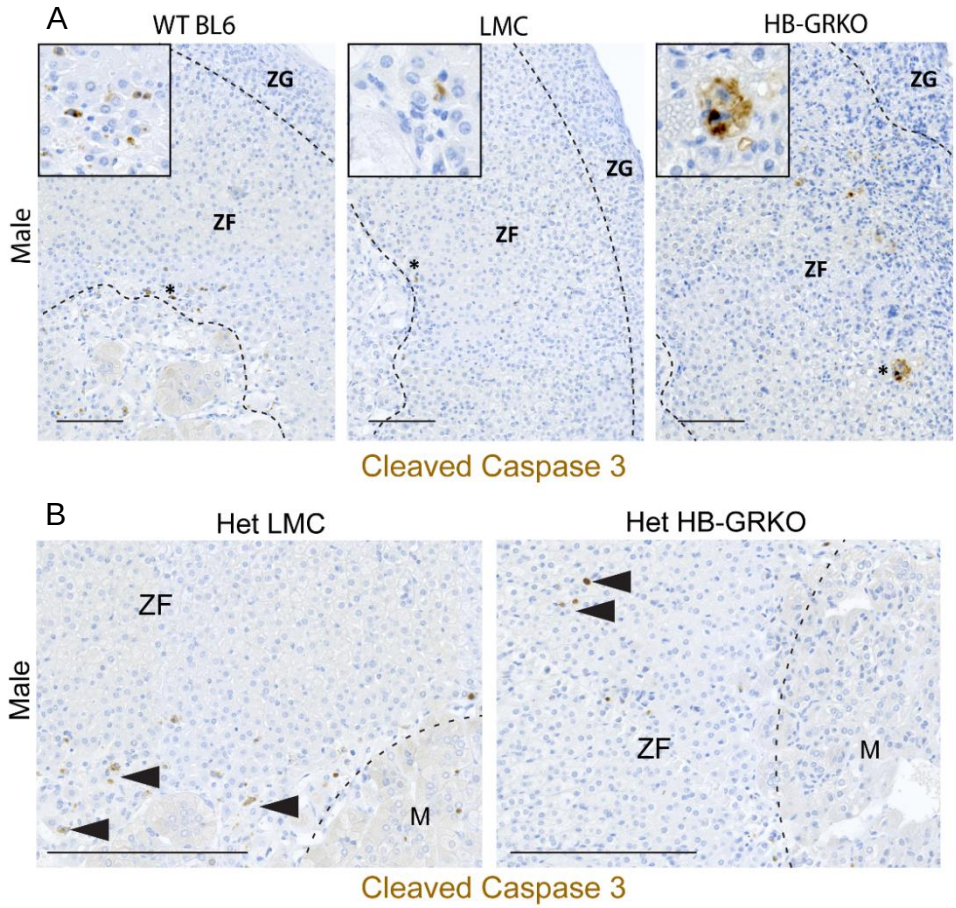


Figure 5-17. HB-GRKO males show aberrant apoptosis throughout the adrenal cortex. ***(A)*** *Cell clearance from the cortex occurs at the cortex-medulla boundary which can be observed in Bl6 male controls via immunohistochemical analysis. HB-GRKO male cleaved caspase protein localisation show abundant cleaved caspase 3 positive cells throughout the cortex. Inserts denoted by (*). Scale bars 50µm.* ***(B)*** *Both Het LMC and Het HB-GRKO show cleaved caspase staing throughout the cortex. Scale bars 100µm Abbreviations; ZG= zona glomerulosa, ZF= zona fasciculata, X=X-zone, M=medulla.*

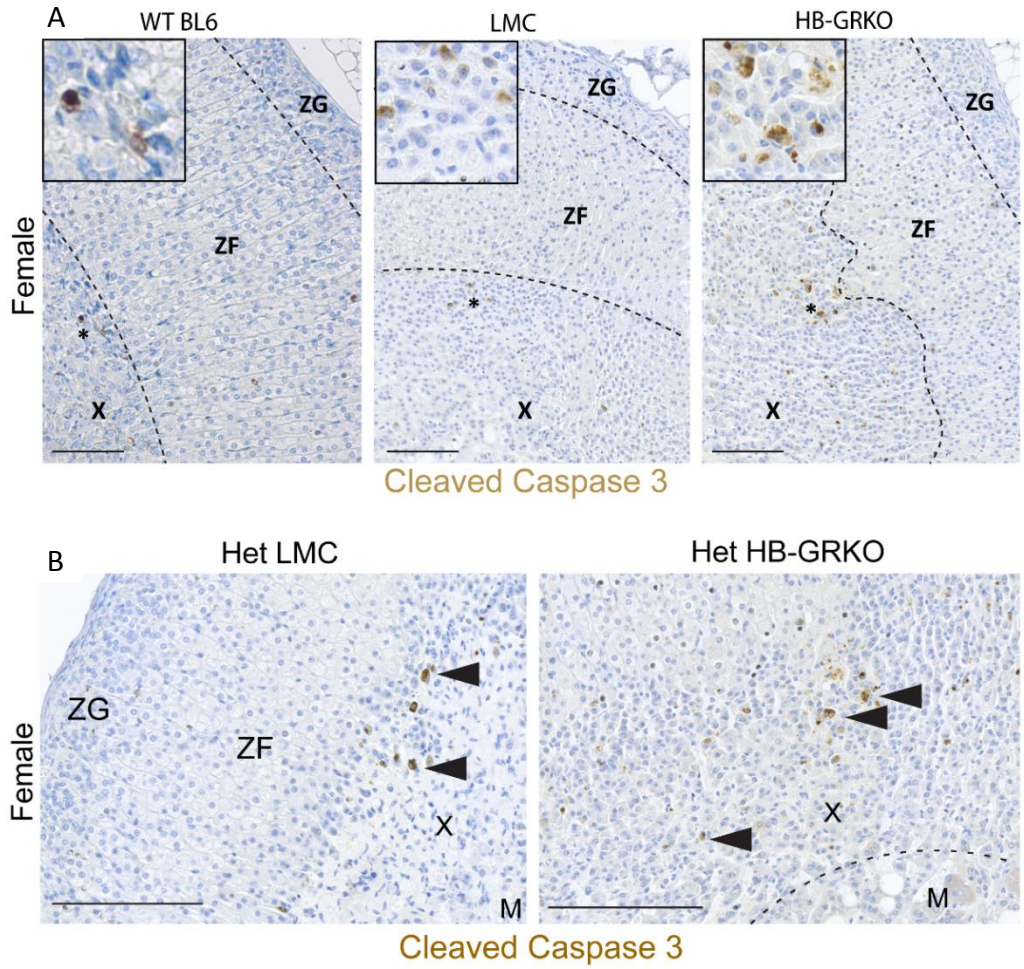


Figure 5-18. HB-GRKO females show aberrant apoptosis throughout the adrenal cortex. ***(A)*** *Cell clearance from the cortex occurs at the cortex-medulla boundary which can be observed in Bl6 female controls via immunohistochemical analysis. HB-GRKO female cleaved caspase protein localisation show abundant cleaved caspase 3 positive cells throughout the cortex. Inserts denoted by (*). Scale bars 50µm.* ***(B)*** *Both Het LMC and Het HB-GRKO show cleaved caspase staing throughout the cortex. Scale bars 100µm. Abbreviations; ZG= zona glomerulosa, ZF= zona fasciculata, X=X-zone, M=medulla.*

In contrast to cleaved caspase staining, no changes in localization of the proliferation marker PCNA was observed in any male or female experimental cohort compared to WT BL6 mice (Fig. 5-19, 5-20). Together, these results show that loss of GR signaling in the hindbrain of both male and female mice, leads to disruption of structure of the adrenal glands. Similar disruption to the adrenal glands of LMCs (who retain hindbrain GR signalling) suggests that the adrenal phenotype is likely a secondary consequence of the stressed behavior (excessive barbering of LMCs) exhibited by HB-GRKO cage-mates. This interpretation is consistent with previous studies linking a chronic stress response to degeneration of the adrenal gland (224).

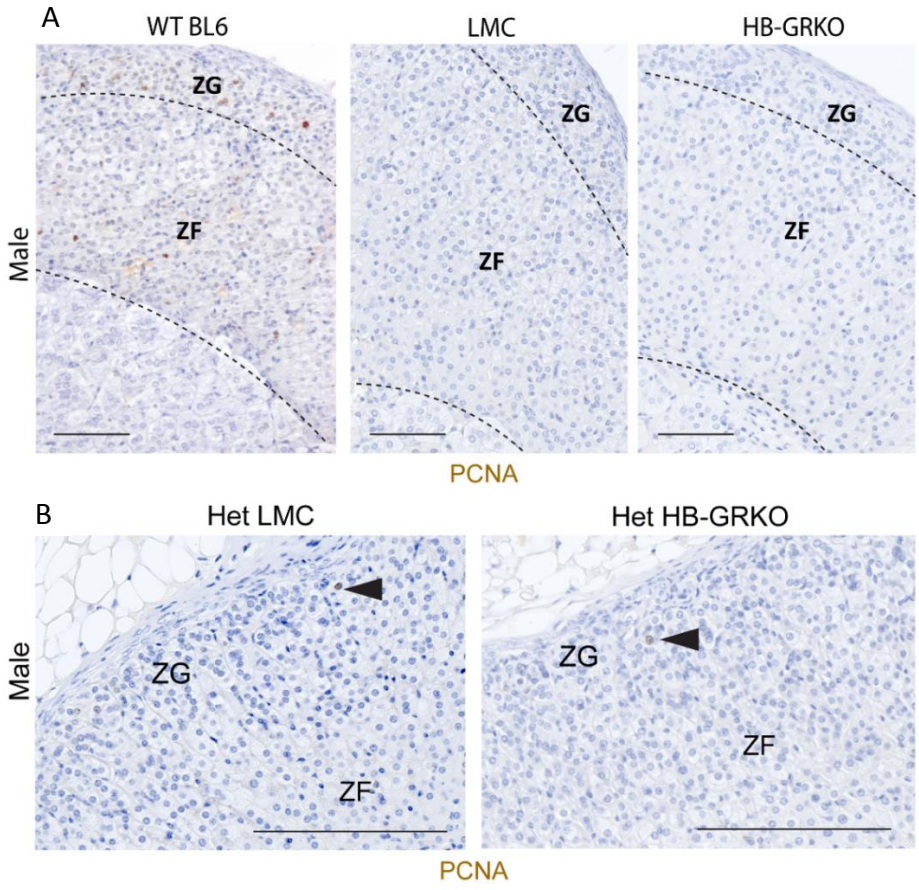


Figure 5-19. LMC and HB-GRKO males show no changes to proliferation. (A, B) *Immunohistochemistry analysis of PCNA revealed no changes in proliferation in any male experimental cohorts compared to WT BL6 controls. Scale bars 100µm. Abbreviations; ZG= zona glomerulosa, ZF= zona fasciculata, X=X-zone.*

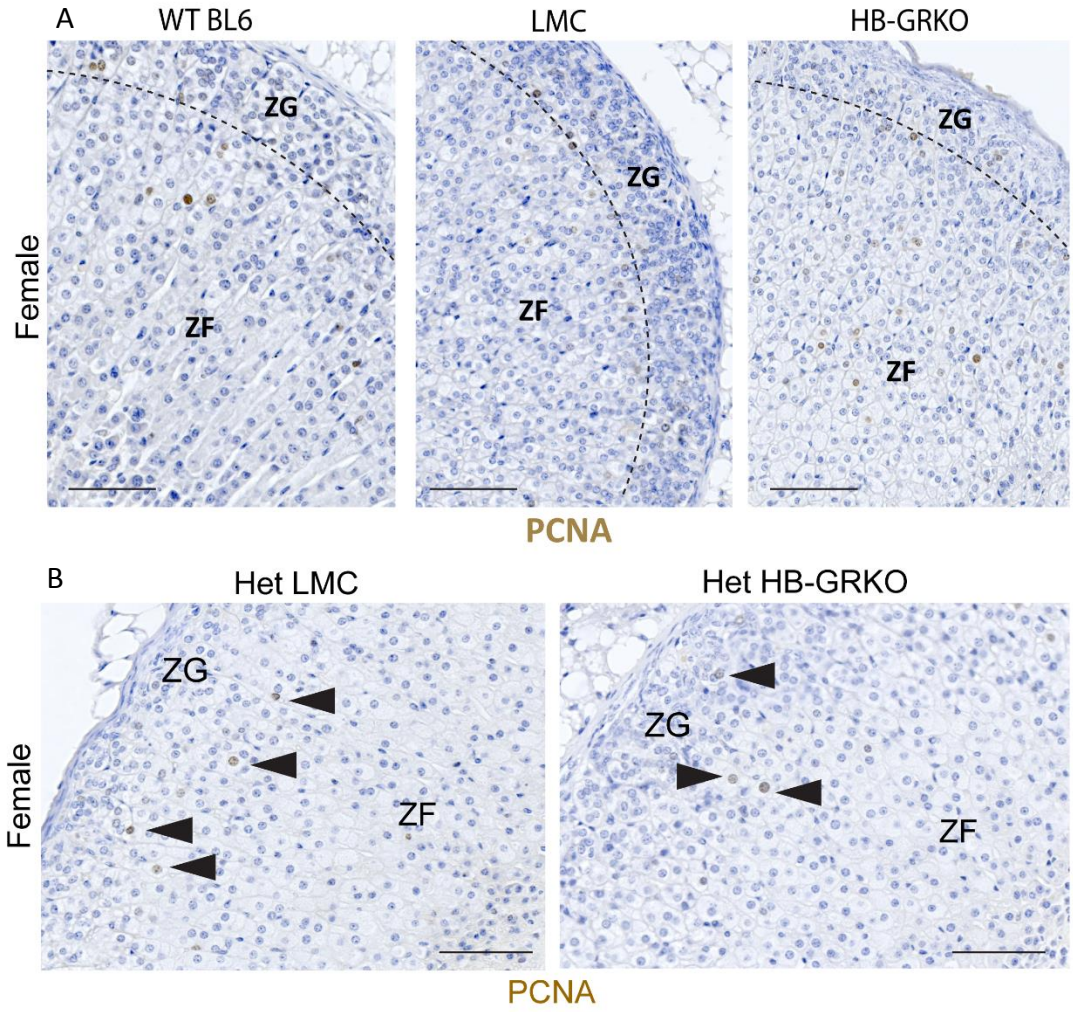


Figure 5-20. LMC and HB-GRKO females show no changes to proliferation. (A, B) *Immunohistochemistry analysis of PCNA revealed no changes in proliferation in any female experimental cohort compared to WT BL6 controls. Scale bars 100µm. Abbreviations; ZG= zona glomerulosa, ZF= zona fasciculata.*

5.3 Discussion

The initial intention of this work was to ablate GR expressing cells in the adrenal cortex, however, due to the inability of the *Cyp11a1*-Cre to target these cells the parameters of the study were re-focused to investigate the impacts of hindbrain GR signalling on the HPA-axis and the adrenal cortex. Ablation of GR in the hindbrain has provided a new animal model for investigating stress and anxiety (253). PCR analysis confirmed recombination of GR in the hindbrain with no off target GR ablation in the adrenal cortex, ensuring any phenotype is a result of hindbrain targeting. These results demonstrate that following GR ablation, stress behaviours presenting as excessive barbering and lack of cage exploration coupled with tendencies of repetitive behaviour can be observed. Additionally, disruption to hindbrain glucocorticoid signalling results in severe adrenal cortex disruption and an elevated stress response that is passed onto littermate controls. Excessive spinal curvature is also observed and could be attributed to the high levels of circulating corticosterone. These results highlight that GR ablation in the hindbrain could provide a novel model to better understand the development of stress and anxiety in patients.

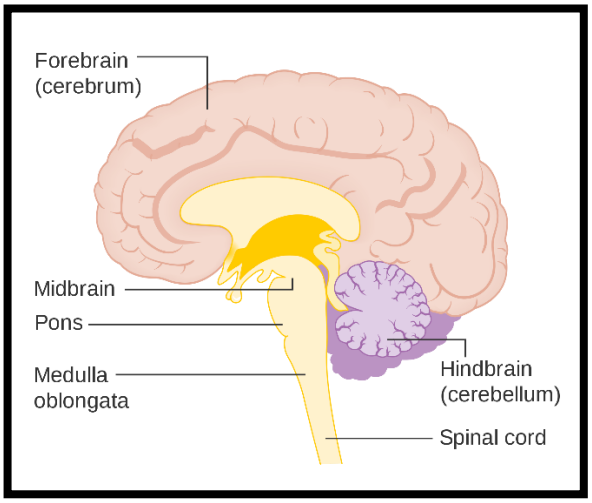


Figure 5-21 Locations of the fore, mid, and hindbrain. *The hindbrain is comprised of the Medulla Oblongata, the Pons (provides the connection between the spinal cord and the rest of the brain), and a large proportion of cranial nerves. The hindbrain is integral in control of autonomous and sub-conscious functions. Adapted from (344).*

Chapter 5: The Cyp11a1-Cre not only permits investigation of the adrenal cortex, but provides a novel model to investigate GR signalling in the hindbrain and its impact on the HPA-axis

Tackling psychiatric conditions such as depression and anxiety has proved difficult with multiple components of the brain being implicated. The initial body of research investigating depression and anxiety focused upon fast acting neurotransmitters such as the impact of serotonin, norepinephrine and dopamine depletion. Despite this, these neurotransmitter systems are short term and cannot account for the progressive and increase in disease severity observed over time in patients with depression or anxiety (345). It was later noted that long term exposure to stress is a leading cause of psychiatric disorders and therefore highlighted glucocorticoids as an important factor for research and future treatment of anxiety and depression (230, 234, 346). The forebrain is well known for its control of cognitive function, so initial models of the role of GR signalling in the brain focused upon this region. Forebrain-specific GR knockout mice generated by Boyle *et al* under a calcium-calmodulin-dependent protein kinase promoter, demonstrated a mouse model with a depressive phenotype and anxiety like behaviours (197). In contrast to this, mice generated by Wei *et al* with an over expression of GR in the forebrain demonstrated an increase in emotional ability in response to applied stressors (199). GR is not limited to the forebrain but has also been identified in the structures of the hindbrain. The hindbrain although known for its role in autonomous regulation, has in recent years, become a region of interest for research into the development of anxiety and depression. Studies conducted by Zhang *et al* demonstrated that glucocorticoids are important for tuning hindbrain stress integration via GR expression in the nucleus of the solitary tract (NTS) (248). Studies have also demonstrated increased glucocorticoids impacting neuronal plasticity and tissue shrinkage suggesting that sustained elevated levels of glucocorticoids cause a reduction in neurogenesis (232). The current challenge facing this field is the two relevant but opposing concepts. The detrimental effects of excessive glucocorticoid levels on the hippocampus seems to require GR to be functioning normally, whereas these same excessive glucocorticoid levels in depression may result from impaired negative feedback inhibition on the HPA axis, which is caused by loss of function of GR. For these reasons, more research is needed into the various regions of the brain expressing GR and their role in depression and anxiety which can be helped through the development of novel models.

Finding an appropriate method to target GR in specific tissues to reduce off target effects is essential to understanding its mode of action. As of late, more research has focused on refined methods to target GR in specific tissues. Using the Smith lab's mouse model of Cre targeted to the mouse *Cyp11a1* locus, highlighted a new way to generate a model of stress. Characterising stress has proved difficult in humans due to the heterogeneity of the behaviours reported in patients with psychiatric disturbances (347). Using mouse models for psychiatric disorders is challenging for these reasons as there is often not a set criteria but a collection of signs and symptoms, made more difficult by the fact that reaction to stress differs in animals and humans. Despite this, a range of indicators of stress behaviour have been well documented in rodents (341, 248, 263). Although not all behaviour observed is identical in humans and rodents, it is indicative of a stressed state and thus enables investigation of the biological mechanisms that may underlie its aetiology, elucidating the neurobiological basis for stress disorders. This model demonstrates a number of characteristics linked to stress behaviour. Initial observations revealed severe hair removal via barbering. Although this is part of normal behaviour for rodents in bonding, dominance and cleaning (264), if performed to excess is indicative of elevated stress levels. HB-GRKO mice had hair loss to the face, head, back and stomach which falls outside normal barbering behaviour (263, 264). In addition to this, monitoring of WT controls and HB-GRKO animals over a five minute period revealed explorative cage behaviour in WT controls, with 16 rears counted and exploration around the cage which falls in line with their normal investigative nature (343). This was not observed in HB-GRKO mice. Little cage exploration was noted and only 2 rears or vertical attempts over the same five-minute period with prolonged periods of excessive digging in the same spot, indicative of a stressed state. This demonstrates that ablation of GR in the hindbrain results in a stress phenotype. An interesting observation of these behaviour was that they did not present themselves until adulthood, with symptoms not becoming apparent before d90-100. The reasons for this still aren't clear, and may be an accumulative effect that progresses and worsens over time.

A study conducted by Webster *et al* demonstrated GR mRNA presence across many structures of the brain, including the hindbrain. They investigated patients with

diagnosed mood disorders and demonstrated GR dysfunction in depression, bipolar and schizophrenia and in much lower mRNA levels compared to 15 healthy individuals. They also noted that there is variability of GR mRNA between each disorder investigated and the anatomical site of GR dysfunction suggesting that GR dysfunction, depending on what region is disrupted, can impact what mood disorder develops (348). There are a number of higher-order autonomic integrative sites in the hindbrain. These include the raphe pallidus, the lateral parabrachial nucleus and the Kölliker-Fuse nucleus, which are responsible for the autonomic response to stressors. Although all of these circuits participate in autonomic integration, their precise role and function in stress-induced responses and behavioural components is not yet defined and requires further research (345). To further investigate the stress phenotype described in this chapter, analysis of GR location and abundance would need to be identified in the hindbrain of HB-GRKO mice and compared with studies such as the one conducted by Webster *et al*. Further to this, a receptor not investigated as part of this study was mineralocorticoid receptor (MR). As described in the literature review of this thesis, glucocorticoids bind to MR with a higher affinity than GR and have been shown to be expressed in the hindbrain (190, 286). With regards to the relation between the MR and GR, both receptors have complementary roles in the regulation of HPA-axis functionality. Additionally, MR has been shown to be an important modulator of stress and influences stress appraisal. However, the primary focus of this research has been on forebrain structures, such as the hippocampus (349). For these reasons, it would be interesting to investigate the impacts on MR as a result of GR ablation in HB-GRKO mice.

In addition to stress behaviours increased spinal curvature was also noted during collection. Kyphosis is the excessive inward concave shape of the spine (350). Studies have described a relationship between the spinal abnormality kyphosis and psychological aspects such as anxiety, depression, and aggression (351). During collection of HB-GRKO mice, it could be seen that these mice have exaggerated spinal curvature. To investigate this further CT scans were used examine bone structure and analyse spinal curvature. CT scans show a significant curvature in HB-GRKO males and females compared to controls. A closer inspection of the spine revealed no fusing

of the vertebrae which suggests that the kyphosis is not due to a developmental problem. The Cobb method of spine measurements confirmed a decreased inward angle compared to controls confirming a significant increase in curvature. Elevated levels of glucocorticoids have been associated with kyphosis and is thought to affect bone density, leading to spinal collapse (300, 302). This data combined with the disruption in the adrenal cortex points to elevated serum corticosterone.

The adrenal is integral in mediating the stress response and defining how it responds to stress and the impacts of dysregulation is essential in understanding and treating psychiatric disorders (109, 317). Mounting evidence shows that patients suffering from stress or depression consistently exhibit hyperactivity of the HPA axis, which results in increased levels of glucocorticoids in these patients (232, 233, 248). For these reasons it was important to ascertain any impacts on the adrenal and determine if disruption to GR in the hindbrain resulted in hyperactivity or disruption to the adrenal cortex. Analysis of adrenal morphology revealed severe disruption in male and female HB-GRKO mice with disorganisation of cortex zones, loss of structure in the ZF and pockets of vacuolisation and hyperplasia. The X-zone in HB-GRKO females was shown to be much larger and had migrated up through the cortex. Surprisingly, Cre-GRflox littermate controls display a similar phenotype. To ensure the phenotype has nothing to do with any disruption of GR that may have occurred through insertion of loxP sites into the gene (195), histology analysis was performed on GR floxed mice from a closed colony and confirmed a normal adrenal. This suggests that the littermate controls are being affected by the HB-GRKO mice. This phenomenon has been described in behavioural studies that demonstrate that stress behaviours in stress-model mice can be passed on to WT cage mates and persist even when the stressed model has been removed (352). For these reasons, an external control was included in the analysis that has had no contact with the stressed mice. HB-GRKO males have an increase in body weight compared to external controls and females have a decrease in body weight. Males also have an increase in adrenal weight, however there were no changes observed in females. These results are fitting of the range of characteristics that are seen in patients with stress disorders and potentially suggests that males and

females differ in their response to stress. Together this data demonstrates that prolonged or chronic stress negatively impacts the adrenal cortex.

Due to results obtained in the chapter three and four of this thesis, describing AR and androgens as important regulators of the adrenal cortex and its markers, analysis of circulating androgens and protein localisation of AR was investigated. The presence of foetal cells in the cortex suggested that there may be a decrease in circulating androgens, however the results show a potential increase. Increasing the sample size could help clarify if this is in fact the case, as results do show a varying range of values. Alternatively, it has been shown in the literature that treatment with endogenous testosterone increases exploratory behaviour in mice and has been shown to help with anxiety (243, 245). This increase in circulating testosterone could potentially be to try to counteract the chronic stress response in HB-GRKO mice.

Changes in circulating glucocorticoids are often used as a measure of stress in many species (282, 289). For these reasons analysis of serum corticosterone was used to determine if HB-GRKO mice had an elevated stress response. Male HB-GRKO and HB-GRKO LMC mice both showed an increase in corticosterone compared to an independent control, revealing an elevated stress response in these animals. Surprisingly, this was not observed in female cohorts. Corticosterone values can vary widely during the oestrous cycle and these females were not collected at a designated stage of the oestrous cycle, which could have contributed to not detecting a difference between the groups. Synchronising of oestrous cycles could potentially provide a more reproducible result. In addition to this, the variability of genotypes per cage could potentially be playing a role. Cages with higher number of Cre+ animals most commonly displayed the most severe phenotype and a less severe phenotype if it was primarily Cre- animals, this could have led to a large variation in samples analysed. These observations coupled with the disruption in heterozygous mice demonstrate strongly that cage mates were impacted by HB-GRKO mice. One of the major drawbacks of the present study was the impact observed in controls. Due to the phenotype observed in all mice, additional external controls had to be included adding to the complexity and additional analysis required to investigate the phenotype observed in knockout mice. The *Cyp11a1*-Cre has been previously used in the lab to

investigate other genes of interest and there have been no identifiable issues with this Cre line prior to, or after breeding to a floxed animal (Appendix, fig. 7-1, a-e). Furthermore, the GR floxed line was also investigated and showed no disruption. This limits the phenotype observed in LMCs to arise from exposure to HB-GRKO mice. For future experimentation, the controls would have to be separated as soon as possible following genotyping. As the phenotype often did not present itself until day 90 onward, this separation would be feasible, although, this separation in itself could create problems. It was noted as part of the study that animals that had a cage with a higher proportion of Cre+ animals often exhibited a more severe phenotype. By separating out the controls, this could be selecting for a more pronounced and exacerbated phenotype. These factors would need to be considered for future use of this model.

Analysis of cortex marker 20 alpha-HSD revealed presence of foetal cells in the cortex of HB-GRKO and HB-GRKO LMC mice, these foetal cells are known to make up the rodent X-zone (95). As discussed in chapters 3 and 4, regression of the X-zone occurs during puberty in males so it should not be present in the adult cortex (23). Females retain the X-zone in adulthood (34, 89), however in this model foetal cells are not constrained in a defined zone, but instead are widely distributed throughout the adrenal cortex. This migration of X-zone cells throughout the cortex have been associated with pathologies such as primary pigmented nodular adrenocortical disease and Cushing's (29). Another cortical marker AKR1B7, known for its role in detoxifying products from cholesterol cleavage was examined (297). Loss of function models show its importance in metabolic function and dysregulation can have serious implications for the adrenal cortex (353). Analysis of AKR1B7 revealed a loss of localisation throughout the cortex in both males and females. Loss of expression could result of toxic products building up in the adrenal cortex and ultimately lead to damage to the cortex.

Normal cell turnover in the adrenal cortex involves the migration of cells from the outer cortex to the cortex-medulla boundary, at the boundary cells undergo apoptosis and removal from the cortex (323). Analysis of cleaved caspase localisation revealed many positive cleaved caspase cells throughout the entire adrenal cortex in HB-GRKO

mice, highlighting considerable cell death and damage to the adrenal cortex. This could be attributed to failed feedback at the hindbrain due to GR ablation, resulting in a maintained stress response and prolonged stimulation of the adrenal cortex. Despite major disruption to the adrenal recognised in all genotypes, an increase in spinal curvature was not present in littermate controls. This suggests that this phenotype is solely caused by ablation of GR from the hindbrain and is not due to a behavioural impact that is being transferred from knockout to control cage mates. The reasons for this are still uncertain, further analysis of bone structure and density could potentially help elucidate the underlying problem or mechanism leading to spinal collapse in HB-GRKO mice.

Previous work looking at chronic stress and its impact on the HPA axis has focused primarily on characterizing chronic stress-induced alterations in the brain. This central focus has occurred in part because the observed brain changes resemble those that are believed to occur in some types of stress-related psychiatric disorders, such as depression and anxiety. Although there is little research in mouse models as to the impact of chronic stress on the adrenals, this has been more extensively researched in rat adrenals. A study conducted by Yvonne *et al* highlighted that chronic stress induced by restraint in male rats, resulted in adrenal hyperplasia and hypertrophy to the adrenal cortex in a zone specific manner (224). This is in line with what is observed in HB-GRKO mice with enlarged adrenals and hyperplasia observed throughout the cortex. This study also noted that there was a decrease in ZG size due to hypoxia. Measurements used in chapter three of this thesis of cortex zones would be useful to determine if there is an impact to specific zone size as a result of chronic stress. Another study investigating chronic stress in female rats during pregnancy noted a resistance to apoptosis (354). This is in contrast to what is observed in the HB-GRKO animals which have apoptosis throughout the adrenal cortex.

According to the World Health Organization, mental disorders will be the second leading cause of disability by the year 2020 (355), so the need for appropriate models to understand psychiatric disorders is ever pressing. This data together further describes the relationship between the GR ablation in the hindbrain and the development of stress. Additionally, it builds on previous literature that describes a

relationship between stress and the dysregulation of the HPA axis. Although further experiments would be needed to identify a specific psychiatric disorder that would be most relevant to the phenotype observed, these results however, demonstrate a new model that could provide new insights into the development and the effects of stress and anxiet

Chapter 6: General Discussion

6.1 Androgen signalling and the male adrenal cortex

Historical studies investigating both male and female rodents have shown that gonadectomy can result an inappropriately maintained X-zone or a re-developed X-zone (34, 36, 38, 53, 95, 356). This highlights that even though it does not produce androgens, the adrenal is responding to androgen signalling and warrants further investigation. Due to the potential relationship between androgen signalling and foetal cells, known to be essential for cortex development and providing stem/progenitor cells, this lead to the hypothesis that androgen signalling is required for normal cortex development and renewal in adulthood in both male and female adrenals. Ablation of androgen receptor from the male adrenal highlighted numerous novel interactions and new roles within the adrenal cortex. Key findings noted the maintenance of the X-zone after puberty and significant morphological differences of the X-zone depending on whether AR or androgens had been targeted. This suggests AR and circulating androgens not only target the adrenal but can do this via independent mechanisms. This result was further strengthened by the loss of AKR1B7 expression following castration but not AR ablation. Although disruption to androgen signalling revealed maintenance or redevelopment of an X-zone, these mice appeared to be overall healthy and made it to adulthood. Experiments to determine if the phenotype progressed with age revealed that prolonged castration resulted in a phenotype similar to what would be observed in aging females, however, ablation of AR alone resulted in an expanded X-zone and development of spindle cell lesions in the outer cortex.

This data highlighted numerous pathways that androgens could be controlling or working in tandem to regulate the adrenal cortex. The consistent renewal of the adrenal cortex and its zones requires is an intricate process with clearly many cell mechanisms and pathways at play. Although there are more studies now beginning to interrogate the mechanisms that regulate the adrenal cortex, there are still many unanswered questions. Changes observed in key cortex regulation genes such as *Sf1* and *Dax1* following AR ablation was noted. These are two key regulators in adrenal homeostasis. Relationships between these genes and AR signalling have been described in other tissues such as the gonads (357, 358) and recognised that AR could directly bind to

Sf1 to block communication, furthermore, showing that androgens can act via *Dax1* to inhibit aromatase expression in breast cancer cell lines (358). Despite this, these relationships have not yet been clearly highlighted in the adrenal cortex. The data suggests androgens are working with these signalling pathways to regulate zonation, which puts the adrenal among the key regulators of the adrenal cortex. An interesting observation of the X-zone during development in Ad-ARKO mice revealed a more pronounced and developed X-zone, where previously it was thought that androgen had no impact on the cortex until puberty (95, 311). Studies examining the process in which the adrenal switches from the foetal to the adult lineage is essential in understanding adrenal development. *Dax1* has been described as a potential adult adrenal enhancer required for the regression of the X-zone and switch to definitive cortex development, this was based on the fact that the X-zone remained in rodent following *Dax1* ablation (61). The same phenotype is also observed in Ad-ARKO mice. This suggests that AR could also be an adult adrenal enhancer, and that the switch from foetal to definitive cortex development has multiple regulators.

Contrary to the literature, AKR1B7 is thought to be regulated by *Sf1* in the adrenal (298), the loss of AKR1B7 following castration suggests that it is in fact regulated by androgens, as is the case for the vas deferens . Following loss of androgen signalling, no apoptotic cells could be observed in the adrenal cortex of these mice. This has been described previously in a global ARKO model (162), but this potential resistance to apoptosis was thought to be from chronic levels of ACTH from the pituitary. Elevated ACTH levels may contribute to the phenotype in the global knockout, however, the results from this project suggests that this phenotype is a result of loss of AR from the adrenal cortex and not the pituitary.

WNT/ β-catenin has been shown to be essential in the appropriate maintenance of the adrenal cortex (30, 46, 68, 251). Due to the presence of the X-zone and spindle cell development in aging animals, this highlighted a potential issue with differentiation. It was hypothesised that there would be disruption to *Wnt4* or β-catenin, however, no changes in transcript were observed. This does not rule out that androgens are not interacting with this pathway. There have been studies conducted that describe β-catenin and AR specific interactions (359). This study highlighted that under certain

conditions, β-catenin and AR can drive the development of prostate cancer. Due to the changes in morphology, key pathways in cortex regulation and HPA-axis noted following disruption to AR and circulating androgens, demonstrates androgen signalling is essential for normal regulation of the adrenal cortex and that the *Cyp11a1*-Cre permitted the novel opportunity to examine the role of androgen signalling specifically within the adrenal cortex. For these reasons, the original hypothesis was accepted and the objectives met.

6.1.1 Future perspectives

This project highlighted numerous new targets and avenues for further investigation. Investigation into functional targets in the WNT/β-catenin pathway (Fz, LRP6, Axin2, TCF/LEF, Naked (a Dvl antagonist), Dkk1, and Rspo) (360-362) following androgen manipulation could help elucidate if androgens could be interacting with this pathway to promote differentiation in the adrenal. Unfortunately, due to time constraints, a suitable β-catenin antibody that worked on the adrenal cortex was not identified. With additional time it would be interesting to see if the localisation of this protein changes upon androgen manipulation. Furthermore, the use of an adrenal cell line such as Y1 cortical cells (363) could potentially have helped investigate additional molecular targets of androgen signalling and their impact on adrenocortical cells following knockdown of AR or treatment with androgens. The isolation of X-zone cells and investigating them in culture under various treatments could help further our understanding in their development or differentiation into definitive cortical cells. This may be difficult to investigate in the mouse due to the size of the adrenal and lack of suitable cell surface markers that could be used in cell sorting. However, adrenals in the rat may be feasible as they are much larger and could potentially be separated easier via fine dissection.

Ad-ARKO mice were kept in a consistent environment which resulted in no situations in which the adrenal becomes challenged. There are periods of stress that can arise through handling during cage cleaning and situations like cage dominance, but these exposures are often short. Determining if the adrenal is able to respond to stressors under conditions such as restraint stress or treatment with dexamethasone would help answer if AR is important in HPA-axis regulation when challenged. Furthermore,

corticosterone levels were only analysed in the morning when corticosterone levels are low. Sampling at different time points throughout the day would allow investigation of the impact of AR signalling not only in stress response but normal cyclical basal levels of corticosterone secretion. Another mouse line that was investigated throughout this thesis, as part of the male and female ARKO projects was an ARE-luciferase mouse line (364). This hoped to identify where androgen signalling was active in the adrenal cortex under normal conditions, castration and hCG treatment. This would permit investigation of adrenal cortex zones and if they respond to androgen signalling. By understanding cell regions and cortex zones in which androgen signalling is active, this could help direct future research regarding specific cellular processes and signalling pathways regulating a particular zone and how they interact with androgens. Despite all animals for these experiments being treated and collected, in the timeframe of this thesis an appropriate antibody to permit localisation of AR signalling was not identified. Investigation via qRT-PCR also did not work and struggled to detect luciferase activity. Future work would see the optimisation of an appropriate antibody and primers.

6.2 Androgen receptor and the female adrenal cortex

Due to the noted relationship between androgen signalling and the X-zone observed in male Ad-ARKO mice, this lead to the hypothesis that androgen signalling is required for normal cortex regulation and regression of the X-zone during pregnancy in female adrenals.

The investigation of androgen receptor ablation in the female was initially to investigate the impact on the female X-zone, however, results obtained through this study suggests that AR plays a vital role in protecting against the development of spindle cell hyperplasia. Key findings during this project highlighted significantly fewer Ad-ARKO females being born compared to male counterparts. No early postnatal developmental issue was identified as a result of partial or complete adrenal AR ablation, however, the development of spindle cell hyperplasia could be observed in early adulthood. This can occur naturally, therefore to observe spindle cells in young mice is rare. Analysis of presence in d80 samples revealed 80% of 2nd generation Ad-

ARKO presented with this phenotype compared to only 20% in littermate age matched controls. Although X-zones are maintained in the female adrenal cortex in adulthood, AR ablation resulted in enlarged X-zones corresponding with an increase in adrenal weight. However, ablation of AR did not result in failed X-zone regression highlighting AR is dispensable for X-zone regression during pregnancy. Despite this, elevated serum corticosterone was revealed in parous Ad-ARKO females, a symptom noted in patients with postpartum depression (327).

This data revealed key differences in males and female in the response to the loss of androgen signalling. Despite AR being required for the regression of the X-zone during puberty in males, results demonstrate AR is not required for the regression during pregnancy. Historical studies have suggested that the X-zone is purely andromimetic (36), however, more recent studies investigating *Prkar1a* (29) and *Thrb* (40), show that the X-zone is under a complex control mechanism that additionally, is sexually dimorphic. It has also been suggested that progesterone could regulate the X-zone in females (34) but the mechanisms as of yet still aren't clear. Despite not regressing during pregnancy, the X-zone does enlarge and can be seen occupying large portions of the adrenal cortex, suggesting an overlapping mechanisms in male and female mice in relation to X-zone regulation. Again, potentially the WNT/β-catenin pathway could be at play.

A major hindrance to this project was the low numbers of 2nd generation Ad-ARKO females being born. Chi squared analysis did highlight significantly less of this genotype being born compared to male littermates. The reasons for this discrepancy are still not clear, there should be no maternal impact as dams in these matings were AR^{flox}. This phenotype has not been documented in global AR knockout models which targets the adrenal (160). This suggests that despite there being significantly fewer born, that it could potentially be an artefact.

The presence of spindle cell hyperplasia in female rodents highlights that AR could be required to protect against the development of this disease. Although it has been noted that many of adrenocortical lesions are benign and cause no further issue in adrenal function, some of these do go on to become malignant (365) and are extremely difficult to treat, knowing what causes progression from benign to malignant is essential for

management of the disease. The spindle cell lesions observed in Ad-ARKO females also stain positive for GR. This study was carried out by Clifton-Bligh *et al* and examined the expression of GR in adrenocortical carcinomas. They suggested that immunohistochemistry of GR localisation in compliment with the Weiss score could help distinguish adrenocortical adenomas from carcinomas, however, the transcriptional activity of GR would need to be further investigated in ACCs to determine if GR is not only a marker but responsible for disease progression. These GR positive spindle cells were also observed in castrated male Ad-ARKOs and aged male Ad-ARKO mice, suggesting that a similar mechanism in males and females is resulting in this spindle cell hyperplasia. Whether this is through interactions with normal homeostasis signalling pathways in the adrenal cortex or a relationship between AR and GR needs further investigation. It has been demonstrated that glucocorticoid receptor expression is negatively regulated by androgen receptor in prostate tumours (203). The fact we see a significant upregulation of GR in the castrated animals during this project could point to a potential mechanism of adrenal tumour development via an AR/GR relationship.

Infiltrating mast cells have also been shown to be important during the development of spindle cell hyperplasia (299, 314, 366). Studies examining infiltrating mast cells in prostate cancer invasion and metastasis describe suppression of AR signals permits the influx of mast cells and the development of metastasis (367). This prompted the examination of *C-kit* transcript in Ad-ARKO females, results however show a significant decrease in *C-kit* transcript. Further investigation would be needed to explore this relationship in the adrenal. However, this change seen in *C-kit* transcript following AR ablation points to a potential mechanisms driving the tumour development in females. The development of spindle cell hyperplasia following disruption to AR and circulating androgens, demonstrates androgen signalling is essential for normal regulation of the female adrenal cortex and prevention of spindle cell development. For these reasons, the original hypothesis was accepted and the objectives met. However, AR signalling is not required for X-zone regression during pregnancy, so this part of the hypothesis was rejected.

6.2.1 Future perspectives

Extended breeding of 2nd generation litters would be needed to ascertain whether or not the discrepancy between genotypes is an artefact. Increasing the samples sizes could clarify this observation. If results do confirm there are significantly less Cre+ females being born, experiments would be needed to investigate the cause. First, examination of litters at d0 would be needed to determine if pups are being born but not surviving and are dying shortly after birth. Alternatively, if the discrepancy is already present in the pups that are born and surviving, an investigation examining embryonic development to see if pups are being reabsorbed or dying in utero could help dissect this problem.

Investigation of mast cell marker in adrenal tissue would be important to investigate the result of *c-kit* transcript downregulation. Attempts were made with markers C-KIT and CD63, however neither antibodies worked in the adrenal tissue. Given more time, it would be important to try these antibodies in adrenals in a different fixative as Bouins can be a difficult fixative to get antibodies to work in due to the cross-linkage of proteins. Attempts made in the Smith group with antibody staining in cryopreserved sections have shown promising results, this could be a potential avenue to examine C-KIT and CD36. LH signalling has also been shown to be an important regulator of the human adrenal cortex. It has been shown following castration in certain mouse strains, that adrenal tumours develop (53). Further studies have shown that surges in LH can switch LH receptor (LHR) on in the rodent adrenal cortex to drive tumour formation (154). Performing transcript and immunohistochemistry analysis would be needed to determine if LHR has potentially became switched on in the adrenal cortex. In addition to LHR interrogation, circulating LH analysis would be needed to determine of Ad-ARKO females have increased circulating LH.

Despite X-zone regression in Ad-ARKO females following pregnancy, it would be interesting to investigate these parous females at later stages post pregnancy. This would be to investigate if AR is required for continued suppression of the X-zone post pregnancy. Additionally, investigation of serum corticosterone at later time points post pregnancy would be needed to determine if corticosterone levels remain elevated in these mice. It was observed that the pups from these litters all died at birth. Rodents with high postpartum corticosterone have been noted to neglect pups (327), which

could potentially be the reason for this. However, the Cre also targets the ovary and could likely be an impact from ovary AR ablation than adrenal.

Additionally, examination of female adrenals from the ARE-luciferase mice for the purposes previously described would provide insight into androgen activity in the female adrenal and if this differs from male adrenals.

6.3 Glucocorticoid receptor and the HPA axis

HPA-axis. The hypothalamic-pituitary-adrenal (HPA) axis is the body's key system when responding to or dealing with stress (368). Intense or chronic bouts of stress have been shown to result in emotional disturbances and hormonal disruption that can ultimately culminate in a psychiatric disorder (229). Due to the known relationship between GR and stress response, this led to the hypothesis that hindbrain GR is essential for normal HPA regulation and adrenal cortex response to stress in males and females.

Key findings from this project revealed that targeting GR in the hindbrain resulted in a stressed phenotype in adult mice that could be transferred to littermates. This presented as moderate to severe barbering of knockout animals and littermate controls. Investigation of individually housed HB-GRKO males and LMC males, showed severe barbering in HB-GRKO mice but not in LMCs. This demonstrated the severe barbering observed in cages regardless of genotype, was stemming from the GR ablated mice. Observations of these same mice under normal conditions prior to collection revealed normal exploratory cage behaviour in LMCs, but anxiety and stress related behaviour in HB-GRKO males. These behaviours manifested as obsessive digging and limited cage exploration. Investigation of adrenal morphology revealed a severely disrupted adrenal cortex in male and females across all genotypes. This result along with the barbering strengthens the argument that HB-GRKO mice were negatively impacting LMCs. Damage to the adrenal cortex in HB-GRKOs and LMCs coincided with disruption to cortical markers AKR1B7 and 20 alpha-HSD, along with aberrant cell death throughout the cortex. Increased spinal curvature was also observed in these animals but were limited to the HB-GRKO mice. These results demonstrate

that ablation of GR in the hindbrain via *Cyp11a1*-Cre provides a new model to investigate stress and anxiety related behaviours and their impact on the adrenal.

Due to the changes observed in GR transcription following adrenal AR ablation in Ad-ARKO mice, the original intention of this part of the project was to examine the role of GR in the adrenal cortex and the impact of ablation on adrenal function and androgen signalling. However, due to the location of GR in epithelial like cells in the adrenal cortex, the *Cyp11a1*-Cre did not target the GR expressing cell types. However, the Cre is active in the hindbrain postnatally (253), and provided a tissue specific ablation without off target effects. Due to the wealth of literature regarding GRs impact in psychiatric disorders and their involvement in the HPA-axis, this provided an opportunity to explore the impact of hindbrain GR signalling on the adrenal cortex in a novel model.

Dysfunctional glucocorticoid signalling has been demonstrated through analysis of patients with depression compared to healthy individuals, which exhibit twice the average daily levels of cortisol outputs (369). Often psychiatric conditions are multifactorial but it is thought to be dysfunctional GR in the brain leading to a failure of the HPA-axis feedback mechanism (286). Understanding the mechanism and using appropriate models to understand the development of anxiety or depression is essential. In an ideal situation, animal models would recapitulate exactly the symptoms displayed in patients, but this is rare. Finding models that best suit a particular condition is often the outcome. With the development of excessive barbering in HB-GRKO animals and the lack of cage exploration, behaviours that are characteristic of animals in a stressed state (263, 352) suggests that HB-GRKO mice could provide a new model for neuropsychiatric research. The impact on littermates, not only in barbering but the resulting impact on their HPA-axis denotes a clear behavioural abnormality in these mice. Further validation of these models however would be required to determine the most appropriate use. There are three criteria are used when validating an animal model, 1. Construct validity (370), incorporates a conceptual analogy to the cause of the human disease i.e. genetic ablation of a target of interest. 2. Face validity (371), incorporates a conceptual analogy to the symptoms of the human disease i.e. behavioural responses and neurophysiological response and 3. Predictive validity (372), incorporates specificity of responses to treatments that are

effective in the human disease i.e. response to a drug treatment. For this model to progress further and be used in neurobiological research, additional work would be required in face validity and predictive validity. An interesting point to mention, is that the phenotype observed in this study did not arise until at least d90 and progressed with age. This could suggest that there is an accumulative effect occurring. The Cre does not become active in the hindbrain until after birth, however, the point in which the Cre becomes active postnatally in the hindbrain is not known. The timing of its expression could help pinpoint the initial development of the phenotype observed and could answer why the phenotype in these animals is not observed during early development and arises in only in adulthood.

As previously mentioned, due to the changes in GR observed in Ad-ARKOs, investigation of androgen signalling in these mice could provide an interesting insight into the relationship between AR and GR in stress. Circulating androgens have been shown in a number of studies to reduce anxiety and enhanced cognition (245, 373-375). With the X-zone data from chapter 3 and 4 and the presence of foetal cells in the adrenal cortex of HB-GRKO mice, this potentially pointed towards disruption to AR in the adrenal or a decrease in circulating androgens. Results highlighted no significant circulating androgens which was opposite to what was expected, with no changes in AR localisation, however, transcript levels of AR were not investigated which would help confirm if there was no impact to AR signalling.

6.3.1 Future perspectives

Further work would be required to investigate the adverse behaviours in HB-GRKO mice and define exactly the behavioural component of this phenotype. Due to the unexpected nature of the phenotype observed in this study. Experiments that examined behaviour were not covered under the labs license, however if this was to be investigated, numerous neurological tests have been designed for rodents. Examples of relevant tests include, reciprocal and social interaction, elevated plus maze, light/dark exploration or Porsolt forced swim test (376). These are tests specifically to define if a genetic manipulation has resulted in a phenotype relating to anxiety or depression.

Due to the behavioural impact on littermate controls, separation of genotypes at an early stage would be needed to avoid influence from knockout animals. Cages that had a higher proportion of Cre+ animals per cage showed some of the worst barbering. Interrogation of hindbrain GR in littermate controls would be important to see if GR was downregulated in these animals, potentially highlighting a feedforward mechanism between HB-GRKO and littermates. Investigation of CRF and ACTH would be important to establish if they are elevated in HB-GRKO mice and are leading to overstimulation of the adrenal leading to cortex damage or if the damage is a result of sustained increases circulating corticosterone. Analysis of spinal measurements revealed an increase in curvature, this phenotype was limited to the Cre+ mice only and was not passed onto littermates. Analysis of bone density in these mice would answer if disruption or increased glucocorticoids is impacting bone density leading to spinal collapse.

The initial hypothesis for this project stated that the *Cyp11a1*-Cre could be used to permit investigation of GR function in the adrenal. Preliminary data showed that the Cre did not target GR expressing cells in the adrenal so was therefore rejected. However, the hindbrain was targeted and permitted the generation of a new hypothesis that hindbrain GR was important in HPA regulation. Due to the disturbances in normal cage and barbering behaviour and adrenal cortex disruption the hypothesis was accepted and the objectives met.

6.4 Final Conclusions

The original hypothesis for this thesis is that AR and GR are important for normal adrenal cortex function during development, X-zone regulation, cortex maintenance, and regulation of the HPA-axis. Through the use of *Cyp11a1*-Cre, ablation of adrenal AR and hindbrain GR, the hypothesis has been proven, describing the following observations:

- AR is required during postnatal development in the male adrenal and is required for X-zone regression and maintenance into adulthood, however is not required X-zone regression during pregnancy.
- Androgen receptor is required for the normal maintenance of cortical marker AKR1B7 and significant morphological differences in X-zone between cohorts demonstrated that androgens are targeting the adrenal cortex independently of their receptor.
- Androgen signalling is required to prevent the development of spindle cell hyperplasia
- GR function in the hindbrain is required to maintain normal HPA axis function and normal behaviour

Additional novel, previously undocumented observations through the course of this thesis have been:

- The presence of AR localisation in embryonic adrenals, showing androgen receptor expression early in embryonic adrenal development.
- The changes in localisation of androgen receptor in the postnatal developing adrenal cortex, showing migration through the adrenal cortex.
- Location of androgen receptor in the human adrenal, showing AR localisation in foetal and adult adrenal.

The *Cyp11a1*-Cre has enabled adrenal specific interrogation of the roles of AR in the adrenal cortex describing new and novel results. This data highlighted relationships with well described signalling pathways and the role of AR in preventing adrenal disease. The *Cyp11a1*-Cre, although not the original intention, provided a new model to investigate stress development and the HPA-axis.

Chapter 7: Appendix

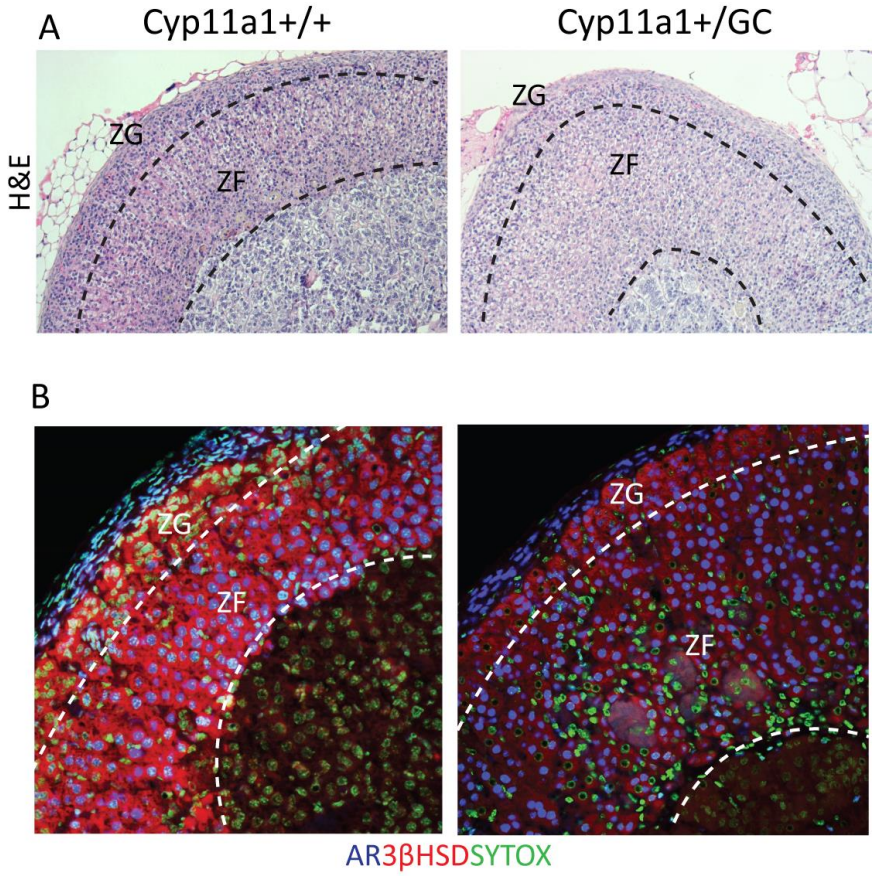


Figure 7-1. There are no morphological differences between Cyp11a1$^{+/+}$ and Cyp11a1$^{+/GC}$ mice. (A) *There are no observable morphological differences as a result of targeting Cre to the Cyp11a1 locus.* ***(B)*** *Immunostaining of androgen receptor and 3ϐHSD shows no impact to steroidogenesis or impact to androgen receptor protein localisation as a result of targeting Cre to the Cyp11a1 locus. Blue; androgen receptor protein, Red; 3ϐHSD protein, Green; sytox counterstain. Scale bars 100µm. Abbreviations; ZG= zona glomerulosa, ZF= zona fasciculata.*

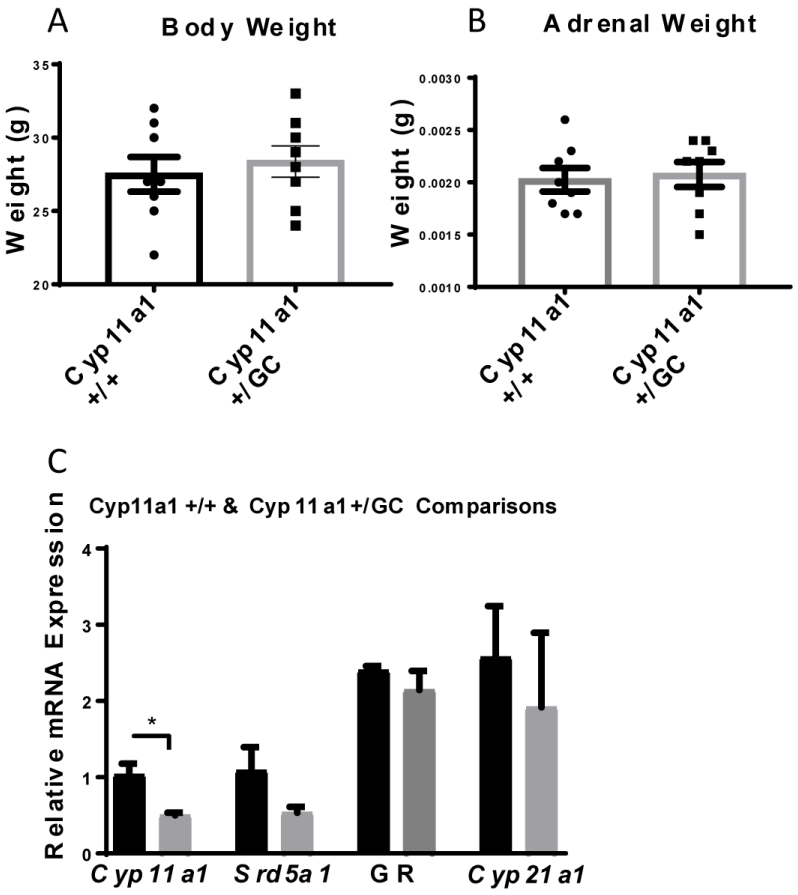


Figure 7-2. There are no weight or transcript differences between Cyp11a1$^{+/+}$ and Cyp11a1$^{+/GC}$ mice. (A, B) *There are no observable differences in body or adrenal weight as a result of targeting Cre to the Cyp11a1 locus.**(C)** Transcript analysis shows a significant downregulation of Cyp11a1 in mice carrying the Cre (one-way ANOVA; n=8, *p<0.05, Tukeys post-hoc analysis, error bars SEM). Despite this, there is no impact on the levels of transcript in the genes of interest as a result of Cre presence.*

8. Bibliography

1. Bolt R, Van Weissenbruch M, Lafeber H, Delemarre-Van de Waal H. Development of the hypothalamic-pituitary-adrenal axis in the fetus and preterm infant. Journal of Pediatric Endocrinology and Metabolism. 2002;15(6):759-70.
2. Kataoka Y, Ikehara Y, Hattori T. Cell proliferation and renewal of mouse adrenal cortex. Journal of Anatomy. 1996;188(Pt 2):375-382.
3. Miller WL, Auchus RJ. The molecular biology, biochemistry, and physiology of human steroidogenesis and its disorders. Endocrine Reviews. 2010;32(1):81-151.
4. Nussdorfer GG. Paracrine control of adrenal cortical function by medullary chromaffin cells. Pharmacological Reviews. 1996;48(4):495-530.
5. Shelton JH, Jones AL. The fine structure of the mouse adrenal cortex and the ultrastructural changes in the zona glomerulosa with low and high sodium diets. The Anatomical Record. 1971;170(2):147-81.
6. Vinson G, Whitehouse B, Goddard C, Sibley C. Comparative and evolutionary aspects of aldosterone secretion and zona glomerulosa function. Journal of Endocrinology. 1979;81(2):55-61.
7. Pantic I, Nesic D, Basailovic M, Cetkovic M, Mazic S, Suzic-Lazic J, et al. Chromatin fractal organization, textural patterns, and circularity of nuclear envelope in adrenal zona fasciculata cells. Microscopy and Microanalysis. 2016;22(6):1120-7.
8. Simpson ER, Waterman MR. Regulation of the synthesis of steroidogenic enzymes in adrenal cortical cells by ACTH. Annual Review of Physiology. 1988;50(1):427-40.
9. Bird IM. In the zone: understanding zona reticularis function and its transformation by adrenarche. Journal of Endocrinology; 2012;29(7):109-111.
10. Dunn TB. Normal and pathologic anatomy of the adrenal gland of the mouse, including neoplasms. Journal of the National Cancer Institute. 1970;44(6):1323-89.
11. Cummings B. The Adrenal Glands Pearson education, Inc2004 [cited 2017 10/08/2017]. Available from: http://www.apsubiology.org/anatomy/2010/2010_Exam_Reviews/Exam_5_Final_Review/CH_16_Th_%20Adrenal_Glands.htm.
12. Jones IC. The adrenal cortex. The adrenal cortex. 1957.
13. Wright N, Voncina D, Morley A. An attempt to demonstrate cell migration from the zona glomerulosa in the prepubertal male rat adrenal cortex. Journal of Endocrinology. 1973;59(3):451-9.
14. Zajicek G, Ariel I, Arber N. The streaming adrenal cortex: direct evidence of centripetal migration of adrenocytes by estimation of cell turnover rate. Journal of Endocrinology. 1986;111(3):477-82.
15. Chang S-P, Morrison HD, Nilsson F, Kenyon CJ, West JD, Morley SD. Cell proliferation, movement and differentiation during maintenance of the adult mouse adrenal cortex. PloS One. 2013;8(12):81-87.
16. Ingle DJ, Higgins GM. Autotransplantation and regeneration of the adrenal gland. Endocrinology. 1938;22(4):458-64.
17. Thomas M, Hornsby PJ. Transplantation of primary bovine adrenocortical cells into scid mice. Molecular and Cellular Endocrinology. 1999;153(1):125-36.
18. Thomas M, Northrup SR, Hornsby PJ. Adrenocortical tissue formed by transplantation of normal clones of bovine adrenocortical cells in scid mice replaces

the essential functions of the animals' adrenal glands. Nature Medicine. 1997;3(9):978-83.
19. Bertholet J. Proliferative activity and cell migration in the adrenal cortex of fetal and neonatal rats: an autoradiographic study. Journal of Endocrinology. 1980;87(1):1-9.
20. Kim AC, Barlaskar FM, Heaton JH, Else T, Kelly VR, Krill KT, et al. In search of adrenocortical stem and progenitor cells. Endocrine Reviews. 2009;30(3):241-63.
21. De Joussineau C, Sahut-Barnola I, Tissier F, Dumontet T, Drelon C, Batisse-Lignier M, et al. mTOR pathway is activated by PKA in adrenocortical cells and participates in vivo to apoptosis resistance in primary pigmented nodular adrenocortical disease (PPNAD). Human Molecular Genetics. 2014;23(20):5418-28.
22. Berthon A, Sahut-Barnola I, Lambert-Langlais S, de Joussineau C, Damon-Soubeyrand C, Louiset E, et al. Constitutive β-catenin activation induces adrenal hyperplasia and promotes adrenal cancer development. Human Molecular Genetics. 2010:ddq029.
23. Hirokawa N, Ishikawa H. Electron microscopic observations on the castration-induced X zone in the adrenal cortex of male mice. Cell and Tissue Research. 1975;162(1):119-30.
24. Ungar F, Stabler TA. 20α-Hydroxysteroid dehydrogenase activity and the X-zone of the female mouse adrenal. Journal of Steroid Biochemistry. 1980;13(1):23-8.
25. Bandiera R, Vidal VP, Motamedi FJ, Clarkson M, Sahut-Barnola I, Von Gise A, et al. WT1 maintains adrenal-gonadal primordium identity and marks a population of AGP-like progenitors within the adrenal gland. Developmental Cell. 2013;27(1):5-18.
26. Wood MA, Acharya A, Finco I, Swonger JM, Elston MJ, Tallquist MD, et al. Fetal adrenal capsular cells serve as progenitor cells for steroidogenic and stromal adrenocortical cell lineages in M. musculus. Development. 2013;140(22):4522-32.
27. King P, Paul A, Laufer E. Shh signaling regulates adrenocortical development and identifies progenitors of steroidogenic lineages. Proceedings of the National Academy of Sciences. 2009;106(50):21185-90.
28. Huang C-CJ, Miyagawa S, Matsumaru D, Parker KL, Yao HH-C. Progenitor cell expansion and organ size of mouse adrenal is regulated by sonic hedgehog. Journal of Endocrinology. 2010;151(3):1119-28.
29. Sahut-Barnola I, De Joussineau C, Val P, Lambert-Langlais S, Damon C, Lefrançois-Martinez A-M, et al. Cushing's syndrome and fetal features resurgence in adrenal cortex–specific prkar1a knockout mice. PLoS One. 2010;6(6):e1000980.
30. Berthon A, Sahut-Barnola I, Lambert-Langlais S, de Joussineau C, Damon-Soubeyrand C, Louiset E, et al. Constitutive β-catenin activation induces adrenal hyperplasia and promotes adrenal cancer development. Human Molecular Genetics. 2010;19(8):1561-76.
31. McEwan PE, Lindop GB, Kenyon CJ. Control of cell proliferation in the rat adrenal gland in vivo by the renin-angiotensin system. American Journal of Physiology-Endocrinology and Metabolism. 1996;271(1):E192-E8.
32. Müller J, Pfeifer U, Dämmrich J. Inhibited autophagic degradation during ACTH-stimulated growth of rat adrenal zona fasciculata. Virchows Archiv B Cell Pathology Zell-pathologie. 1986;52(1):429-41.

33. Leśniewska B, Nowak K, Malendowicz L. Dexamethasone-induced adrenal cortex atrophy and recovery of the gland from partial, steroid-induced atrophy. Experimental and Clinical Endocrinology & Diabetes. 1992;100(06):133-9.
34. Holmes P, Dickson A. X-zone degeneration in the adrenal glands of adult and immature female mice. Journal of Anatomy. 1971;108(Pt 1):159.
35. Suto J-i. Quantitative trait locus mapping of genes associated with vacuolation in the adrenal X-zone of the DDD/Sgn inbred mouse. BMC Genetics. 2012;13(1):95.
36. Starkey WF, Schmidt JR EC. The effect of testosterone-propionate on the x-zone of the mouse adrenal. Journal of Endocrinology. 1938;23(3):339-44.
37. Daughaday W. A comparison of the X-zone of the adrenal cortex in two inbred strains of mice. Cancer Research. 1941;1(11):883-5.
38. Janat M, Shire J. The adrenal X-zone of mice: genetic analysis of its development with recombinant-inbred strains. Experimental Biology. 1986;46(4):217-21.
39. Huang C-CJ, Forrest D. Thyroid Hormone Receptor β1-Mediated Adrenal Remodeling. HPT Axis Biology and Thyroid Hormone Action: Journal of Endocrinology;2014;23(7):0471-0480.
40. Huang C-CJ, Kraft C, Moy N, Ng L, Forrest D. A novel population of inner cortical cells in the adrenal gland that displays sexually dimorphic expression of thyroid hormone receptor-β1. Journal of Endocrinology. 2015;156(6):2338-48.
41. Speiser PW, White PC. Congenital adrenal hyperplasia. New England Journal of Medicine. 2003;349(8):776-88.
42. White PC, Speiser PW. Congenital adrenal hyperplasia due to 21-hydroxylase deficiency 1. Endocrine Reviews. 2000;21(3):245-91.
43. Ching S, Vilain E. Targeted disruption of sonic hedgehog in the mouse adrenal leads to adrenocortical hypoplasia. Genesis. 2009;47(9):628-37.
44. Laufer E, Kesper D, Vortkamp A, King P. Sonic hedgehog signaling during adrenal development. Molecular and Cellular Endocrinology. 2012;351(1):19-27.
45. Leonardo Guastia WCCS, Tristan McKaya, Richard Groseb, Peter J. Kinga. FGF signalling through Fgfr2 isoform IIIb regulates adrenal cortex development. Molecular and Cellular Endocrinology. 2013;371(1):182-8.
46. Berthon A, Martinez A, Bertherat J, Val P. Wnt/β-catenin signalling in adrenal physiology and tumour development. Molecular and Cellular Endocrinology. 2012;351(1):87-95.
47. MacDonald BT, Tamai K, He X. Wnt/β-catenin signaling: components, mechanisms, and diseases. Developmental Cell. 2009;17(1):9-26.
48. Jung SJ, Oh S, Lee GT, Chung J, Min K, Yoon J, et al. Clinical significance of Wnt/β-catenin signalling and androgen receptor expression in prostate cancer. The World Journal of Men's Health. 2013;31(1):36-46.
49. de Joussineau C, Sahut-Barnola I, Levy I, Saloustros E, Val P, Stratakis CA, et al. The cAMP pathway and the control of adrenocortical development and growth. Molecular and Cellular Endocrinology. 2012;351(1):28-36.
50. Fottner C, Hoeflich A, Wolf E, Weber M. Role of the insulin-like growth factor system in adrenocortical growth control and carcinogenesis. Hormone and Metabolic research. 2004;36(06):397-405.
51. Belgorosky A, Baquedano MS, Guercio G, Rivarola MA. Expression of the IGF and the aromatase/estrogen receptor systems in human adrenal tissues from early

infancy to late puberty: implications for the development of adrenarche. Reviews in Endocrine and Metabolic Disorders. 2009;10(1):51.
52. Hofland J, de Jong FH. Inhibins and activins: their roles in the adrenal gland and the development of adrenocortical tumors. Molecular and Cellular Endocrinology. 2012;359(1):92-100.
53. Beuschlein F, Looyenga BD, Bleasdale SE, Mutch C, Bavers DL, Parlow AF, et al. Activin induces x-zone apoptosis that inhibits luteinizing hormone-dependent adrenocortical tumor formation in inhibin-deficient mice. Molecular and Cellular Biology. 2003;23(11):3951-64.
54. Itoh F, Watabe T, Miyazono K, editors. Roles of TGF-β family signals in the fate determination of pluripotent stem cells. Seminars in Cell & Developmental Biology; 2014; 32: 98-106).
55. Schillebeeckx M, Pihlajoki M, Gretzinger E, Yang W, Thol F, Hiller T, et al. Novel markers of gonadectomy-induced adrenocortical neoplasia in the mouse and ferret. Molecular and Cellular Endocrinology. 2015;399:122-30.
56. Bielinska M, Kiiveri S, Parviainen H, Mannisto S, Heikinheimo M, Wilson D. Gonadectomy-induced adrenocortical neoplasia in the domestic ferret (Mustela putorius furo) and laboratory mouse. Veterinary Pathology. 2006;43(2):97-117.
57. Ozisik G, Achermann JC, Meeks JJ, Jameson JL. SF1 in the development of the adrenal gland and gonads. Hormone Research in Paediatrics. 2003;59(Suppl. 1):94-8.
58. Xing Y, Finco I, Hammer GD. Phosphorylation of Nr5a1 (Sf1) regulates mouse adrenal development. Molecular and Cellular Endocrinology. 2015; 344:275-282.
59. Achermann JC, Jeffs B, Jameson JL. SF-1 and DAX-1 in adrenal development and pathology. Adrenal Disease in Childhood. 2: Karger Publishers; 2000. p. 1-23.
60. Lalli E, Melner MH, Stocco DM, Sassone-Corsi P. DAX-1 blocks steroid production at multiple levels. Journal of Endocrinology. 1998; 139(10):4237-43.
61. Scheys JO, Heaton JH, Hammer GD. Evidence of adrenal failure in aging Dax1-deficient mice. Journal of Endocrinology. 2011;152(9):3430-9.
62. Val P, Martinez-Barbera J-P, Swain A. Adrenal development is initiated by Cited2 and Wt1 through modulation of Sf-1 dosage. Development. 2007;134(12):2349-58.
63. Parviainen H, Kiiveri S, Bielinska M, Rahman N, Huhtaniemi IT, Wilson DB, et al. GATA transcription factors in adrenal development and tumors. Molecular and Cellular Endocrinology. 2007;265:17-22.
64. Jimenez P, Saner K, Mayhew B, Rainey WE. GATA-6 is expressed in the human adrenal and regulates transcription of genes required for adrenal androgen biosynthesis. Journal of Endocrinology. 2003;144(10):4285-8.
65. Marjut Pihlajoki EG, Rebecca Cochran, Antti Kyrönlahti, Anja Schrade, Theresa Hiller, Laura Sullivan, Michael Shoykhet, Erica L. Schoeller, Michael D. Brooks, Markku Heikinheimo, and David B. conditional mutagenesis of gata6 in SF1-positive cells causes gonadal-like differentiation in the adrenal cortex of mice. Journal of Endocrinology. 2013; 154(5): 1754–1767.
66. Stone DM, Hynes M, Armanini M, Swanson TA. The tumour-suppressor gene patched encodes a candidate receptor for Sonic hedgehog. Nature. 1996;384(6605):129.

67. Finco I, LaPensee CR, Krill KT, Hammer GD. Hedgehog signaling and steroidogenesis. Annual Review of Physiology. 2015;77:105-29.
68. Kim AC, Reuter AL, Zubair M, Else T, Serecky K, Bingham NC, et al. Targeted disruption of β-catenin in Sf1-expressing cells impairs development and maintenance of the adrenal cortex. Development. 2008;135(15):2593-602.
69. Juhlin CC, Goh G, Healy JM, Fonseca AL, Scholl UI, Stenman A, et al. Whole-exome sequencing characterizes the landscape of somatic mutations and copy number alterations in adrenocortical carcinoma. The Journal of Clinical Endocrinology & Metabolism. 2015;100(3):E493-E502.
70. Bernichtein S, Alevizaki M, Huhtaniemi I. Is the adrenal cortex a target for gonadotropins? Trends in Endocrinology & Metabolism. 2008;19(7):231-8.
71. Parker LN, Sack J, Fisher DA, Odell WD. The adrenarche: prolactin, gonadotropins, adrenal androgens, and cortisol. The Journal of Clinical Endocrinology & Metabolism. 1978;46(3):396-401.
72. Pitetti J-L, Calvel P, Romero Y, Conne B, Truong V, Papaioannou MD, et al. Insulin and IGF1 receptors are essential for XX and XY gonadal differentiation and adrenal development in mice. PLoS One. 2013;9(1):e1003160.
73. Penhoat A, Rainey W, Viard I, Saez J. Regulation of adrenal cell-differentiated functions by growth factors. Hormone Research in Paediatrics. 1994;42(1-2):39-43.
74. Watabe T, Miyazono K. Roles of TGF-[beta] family signaling in stem cell renewal and differentiation. Cell Research. 2009;19(1):103.
75. Guasti L, Sze WC, McKay T, Grose R, King PJ. FGF signalling through Fgfr2 isoform IIIb regulates adrenal cortex development. Molecular and Cellular Endocrinology. 2013;371(1):182-8.
76. Ruggiero C, Doghman M, Lalli E. How genomic studies have improved our understanding of the mechanisms of transcriptional regulation by NR5A nuclear receptors. Molecular and Cellular Endocrinology. 2015;408:138-44.
77. Sadovsky Y, Crawford PA, Woodson KG, Polish JA, Clements MA, Tourtellotte LM, et al. Mice deficient in the orphan receptor steroidogenic factor 1 lack adrenal glands and gonads but express P450 side-chain-cleavage enzyme in the placenta and have normal embryonic serum levels of corticosteroids. Proceedings of the National Academy of Sciences. 1995;92(24):10939-43.
78. Lichtenauer UD, Duchniewicz M, Kolanczyk M, Hoeflich A, Hahner S, Else T, et al. Pre-B-cell transcription factor 1 and steroidogenic factor 1 synergistically regulate adrenocortical growth and steroidogenesis. Journal of Endocrinology. 2007;148(2):693-704.
79. Beuschlein F, Mutch C, Bavers DL, Ulrich-Lai YM, Engeland WC, Keegan C, et al. Steroidogenic factor-1 is essential for compensatory adrenal growth following unilateral adrenalectomy. Journal of Endocrinology. 2002;143(8):3122-35.
80. Parker KL. The roles of steroidogenic factor 1 in endocrine development and function. Molecular and Cellular Endocrinology. 1998;145(1):15-20.
81. Doghman M, Karpova T, Rodrigues GA, Arhatte M, De Moura J, Cavalli LR, et al. Increased steroidogenic factor-1 dosage triggers adrenocortical cell proliferation and cancer. Molecular Endocrinology. 2007;21(12):2968-87.
82. Figueiredo BC, Cavalli LR, Pianovski MAD, Lalli E, Sandrini R, Ribeiro RC, et al. Amplification of the steroidogenic factor 1 gene in childhood adrenocortical tumors. The Journal of Clinical Endocrinology & Metabolism. 2005;90(2):615-9.

83. Bandiera R, Sacco S, Vidal VP, Chaboissier M-C, Schedl A. Steroidogenic organ development and homeostasis: a WT1-centric view. Molecular and Cellular Endocrinology. 2015;408:145-55.
84. Zubair M, Ishihara S, Oka S, Okumura K, Morohashi K-i. Two-step regulation of Ad4BP/SF-1 gene transcription during fetal adrenal development: initiation by a Hox-Pbx1-Prep1 complex and maintenance via autoregulation by Ad4BP/SF-1. Molecular and Cellular Biology. 2006;26(11):4111-21.
85. Kiiveri S, Liu J, Westerholm-Ormio M, Narita N, Wilson DB, Voutilainen R, et al. Differential expression of GATA-4 and GATA-6 in fetal and adult mouse and human adrenal tissue. Jounal of Endocrinology. 2002;143(8):3136-43.
86. Pihlajoki M, Gretzinger E, Cochran R, Kyrönlahti A, Schrade A, Hiller T, et al. Conditional mutagenesis of Gata6 in SF1-positive cells causes gonadal-like differentiation in the adrenal cortex of mice. Journal of Endocrinology. 2013;154(5):1754-67.
87. Morohashi Ki. The ontogenesis of the steroidogenic tissues. Genes to Cells. 1997;2(2):95-106.
88. Mesiano S, Jaffe RB. Developmental and functional biology of the primate fetal adrenal cortex. Endocrine Reviews. 1997;18(3):378-403.
89. Shire J, Spickett S. A strain difference in the time and mode of regression of the adrenal X zone in female mice. General and Comparative Endocrinology. 1968;11(2):355-65.
90. Uotila UU. The early embryological development of the fetal and permanent adrenal cortex in man. The Anatomical Record. 1940;76(2):183-203.
91. Crowder RE. The development of the adrenal gland in man, with special reference to origin and ultimate location of cell types and evidence in favor of the" cell migration" theory: Carnegie Institution; 1957.
92. Richardson D, Joyce D, Symonds EM. Frozen human semen: Springer Science & Business Media; 1980; Vol 4.
93. Zubair M, Parker KL, Morohashi K-i. Developmental links between the fetal and adult zones of the adrenal cortex revealed by lineage tracing. Molecular and Cellular Biology. 2008;28(23):7030-40.
94. Yu RN, Ito M, Saunders TL, Camper SA, Jameson JL. Role of Ahch in gonadal development and gametogenesis. Nature Genetics. 1998;20(4).
95. Siuda S. The occurrence of X-zone in the adrenal glands of two rodent species. Acta Theriologica. 1973;18(25):471-80.
96. Muscatelli F, Strom TM, Walker AP, Zanaria E. Mutations in the DAX-1 gene give rise to both X-linked adrenal hypoplasia congenita and hypogonadotropic hypogonadism. Nature. 1994;372(6507):672.
97. Zanaria E, Muscatelli F, Bardoni B, Strom TM. An unusual member of the nuclear hormone receptor superfamily responsible for X-linked adrenal hypoplasia congenita. Nature. 1994;372(6507):635.
98. Deanesly R, Parkes A. Multiple activities of androgenic compounds. Experimental Physiology. 1937;26(4):393-402.
99. Jones I. The action of testosterone on the adrenal cortex of the hypophysectomized, prepuberally castrated male mouse. Journal of Endocrinology. 1949;44(5):427-38.

100. Koritz SB, Kumar AM. On the mechanism of action of the adrenocorticotrophic Hormone. The stimulation of the activity of enzymes involved in pregnenolone synthesis. Journal of Biological Chemistry. 1970;245(1):152-9.
101. Häggström M. Diagram of the pathways of human steroidogenesis. WikiJournal of Medicine 1 (1). 2014.
102. Mulatero P, Curnow K, Aupetit-Faisant B, Foekling M, Gomez-Sanchez C, Veglio F, et al. Recombinant CYP11B genes encode enzymes that can catalyze conversion of 11-deoxycortisol to cortisol, 18-hydroxycortisol, and 18-oxocortisol. The Journal of Clinical Endocrinology & Metabolism. 1998;83(11):3996-4001.
103. Lachance Y, Labrie C, Simard J, Dumont M, De Launoit Y, Guérin S, et al. Characterization of human 3 beta-hydroxysteroid dehydrogenase/delta 5-delta 4-isomerase gene and its expression in mammalian cells. Journal of Biological Chemistry. 1990;265(33):20469-75.
104. White PC. Disorders of aldosterone biosynthesis and action. New England Journal of Medicine. 1994;331(4):250-8.
105. Booth RE, Johnson JP, Stockand JD. Aldosterone. Advances in Physiology Education. 2002;26(1):8-20.
106. Sanderson JT. The steroid hormone biosynthesis pathway as a target for endocrine-disrupting chemicals. Toxicological Sciences. 2006;94(1):3-21.
107. Suzuki T, Sasano H, Takeyama J, Kaneko C, Freije WA, Carr BR, et al. Developmental changes in steroidogenic enzymes in human postnatal adrenal cortex: immunohistochemical studies. Clinical Endocrinology. 2000;53(6):739-47.
108. Kater CE, Biglieri EG. Disorders of steroid 17 alpha-hydroxylase deficiency. Endocrinology and Metabolism Clinics of North America. 1994;23(2):341-57.
109. Dallman MF, Akana SF, Levin N, Walker CD, Bradbury MJ, Suemaru S, et al. Corticosteroids and the control of function in the hypothalamo-pituitary-adrenal (HPA) axis. Annals of the New York Academy of Sciences. 1994;746(1):22-31.
110. Auchus RJ, Lee TC, Miller WL. Cytochrome b 5 augments the 17, 20-lyase activity of human P450c17 without direct electron transfer. Journal of Biological Chemistry. 1998;273(6):3158-65.
111. Van Weerden W, Bierings H, Van Steenbrugge G, De Jong F, Schröder F. Adrenal glands of mouse and rat do not synthesize androgens. Life Sciences. 1992;50(12):857-61.
112. Noordam C, Dhir V, McNelis JC, Schlereth F, Hanley NA, Krone N, et al. Inactivating PAPSS2 mutations in a patient with premature pubarche. New England Journal of Medicine. 2009;360(22):2310-8.
113. Reiter EO, Fuldauer VG, Root AW. Secretion of the adrenal androgen, dehydroepiandrosterone sulfate, during normal infancy, childhood, and adolescence, in sick infants, and in children with endocrinologic abnormalities. The Journal of Pediatrics. 1977;90(5):766-70.
114. Lois K, Kassi E, Prokopiou M, Chrousos G. Adrenal androgens and aging. 2000.
115. Turcu A, Smith JM, Auchus R, Rainey WE. Adrenal Androgens and Androgen Precursors—Definition, synthesis, regulation and physiologic actions. comprehensive physiology. 2014.
116. Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schütz G, Umesono K, et al. The nuclear receptor superfamily: the second decade. Cell. 1995;83(6):835-9.

117. Burris TP, Busby SA, Griffin PR. Targeting orphan nuclear receptors for treatment of metabolic diseases and autoimmunity. Chemistry & Biology. 2012;19(1):51-9.
118. Levin ER, Hammes SR. Nuclear receptors outside the nucleus: extranuclear signalling by steroid receptors. Nature Reviews Molecular Cell Biology. 2016;17(12):783-97.
119. Migeon BR, Brown TR, Axelman J, Migeon CJ. Studies of the locus for androgen receptor: localization on the human X chromosome and evidence for homology with the Tfm locus in the mouse. Proceedings of the National Academy of Sciences. 1981;78(10):6339-43.
120. Simental JA, Sar M, Lane MV, French FS, Wilson EM. Transcriptional activation and nuclear targeting signals of the human androgen receptor. Journal of Biological Chemistry. 1991;266(1):510-8.
121. Lorente D, Mateo J, Zafeiriou Z, Smith AD, Sandhu S, Ferraldeschi R, et al. Switching and withdrawing hormonal agents for castration-resistant prostate cancer. Nature Reviews Urology. 2015;12(1):37-47.
122. Jenster G, van der Korput HA, van Vroonhoven C, van der Kwast TH, Trapman J, Brinkmann AO. Domains of the human androgen receptor involved in steroid binding, transcriptional activation, and subcellular localization. Molecular Endocrinology. 1991;5(10):1396-404.
123. Jenster G, van der Korput HA, Trapman J, Brinkmann AO. Identification of two transcription activation units in the N-terminal domain of the human androgen receptor. Journal of Biological Chemistry. 1995;270(13):7341-6.
124. Berg JM. DNA binding specificity of steroid receptors. Cell. 1989;57(7):1065-8.
125. Schoenmakers E, Philippe A, Verrijdt G, Peeters B, Verhoeven G, Rombauts W, et al. Differential DNA binding by the androgen and glucocorticoid receptors involves the second Zn-finger and a C-terminal extension of the DNA-binding domains. Biochemical Journal. 1999;341(3):515-21.
126. Loy C, Sim K, Yong E. Filamin-A fragment localizes to the nucleus to regulate androgen receptor and coactivator functions. Proceedings of the National Academy of Sciences. 2003;100(8):4562-7.
127. Castoria G, D'Amato L, Ciociola A, Giovannelli P, Giraldi T, Sepe L, et al. Androgen-induced cell migration: role of androgen receptor/filamin A association. PloS One. 2011;6(2):e17218.
128. He B, Minges JT, Lee LW, Wilson EM. The FXXLF motif mediates androgen receptor-specific interactions with coregulators. Journal of Biological Chemistry. 2002;277(12):10226-35.
129. Picard D, Khursheed B, Garabedian MJ, Fortin MG, Lindquist S, Yamamoto KR. Reduced levels of hsp90 compromise steroid receptor action in vivo. Nature. 1990;348(6297):166-8.
130. Beato M, Truss M, Chavez S. Control of transcription by steroid hormones. Annals of the New York Academy of Sciences. 1996;784(1):93-123.
131. Georget V, Lobaccaro J, Terouanne B, Mangeat P, Nicolas J-C, Sultan C. Trafficking of the androgen receptor in living cells with fused green fluorescent protein–androgen receptor. Molecular and Cellular Endocrinology. 1997;129(1):17-26.

132. Roche PJ, Hoare SA, Parker MG. A consensus DNA-binding site for the androgen receptor. Molecular Endocrinology. 1992;6(12):2229-35.
133. Moehren U, Denayer S, Podvinec M, Verrijdt G, Claessens F. Identification of androgen-selective androgen-response elements in the human aquaporin-5 and Rad9 genes. Biochemical Journal. 2008;411(3):679-86.
134. McKenna NJ, Lanz RB, O'malley BW. Nuclear receptor coregulators: cellular and molecular biology. Endocrine Reviews. 1999;20(3):321-44.
135. Estrada M, Espinosa A, Müller M, Jaimovich E. Testosterone stimulates intracellular calcium release and mitogen-activated protein kinases via a G protein-coupled receptor in skeletal muscle cells. Jounral of Endocrinology. 2003;144(8):3586-97.
136. Kang HY, Cho CL, Huang KL, Wang JC, Hu YC, Lin HK, et al. Nongenomic androgen activation of phosphatidylinositol 3-Kinase/Akt signaling pathway in MC3T3-E1 Osteoblasts. Journal of Bone and Mineral Research. 2004;19(7):1181-90.
137. Gill A, Jamnongjit M, Hammes SR. Androgens promote maturation and signaling in mouse oocytes independent of transcription: a release of inhibition model for mammalian oocyte meiosis. Molecular Endocrinology. 2004;18(1):97-104.
138. Lin H-K, Yeh S, Kang H-Y, Chang C. Akt suppresses androgen-induced apoptosis by phosphorylating and inhibiting androgen receptor. Proceedings of the National Academy of Sciences. 2001;98(13):7200-5.
139. Gorczynska E, Handelsman DJ. Androgens rapidly increase the cytosolic calcium concentration in Sertoli cells. Journal of Endocrinology. 1995;136(5):2052-9.
140. Benten WPM, Lieberherr M, Stamm O, Wrehlke C, Guo Z, Wunderlich F. Testosterone signaling through internalizable surface receptors in androgen receptor-free macrophages. Molecular Biology of the Cell. 1999;10(10):3113-23.
141. Benten WPM, Lieberherr M, Giese G, Wrehlke C, Stamm O, Sekeris CE, et al. Functional testosterone receptors in plasma membranes of T cells. The FASEB Journal. 1999;13(1):123-33.
142. Sarwar M, Sandberg S, Abrahamsson P-A, Persson JL, editors. Protein kinase A (PKA) pathway is functionally linked to androgen receptor (AR) in the progression of prostate cancer. Urologic Oncology: Seminars and Original Investigations; 2014: Elsevier. (Vol. 32, No. 1, pp. 25-e1).
143. Stein MN, Patel N, Bershadskiy A, Sokoloff A, Singer EA. Androgen synthesis inhibitors in the treatment of castration-resistant prostate cancer. Asian Journal of Andrology. 2014;16(3):387.
144. Wang Q, Li W, Zhang Y, Yuan X, Xu K, Yu J, et al. Androgen receptor regulates a distinct transcription program in androgen-independent prostate cancer. Cell. 2009;138(2):245-56.
145. Sen A, Prizant H, Hammes SR. Understanding extranuclear (nongenomic) androgen signaling: what a frog oocyte can tell us about human biology. Steroids. 2011;76(9):822-8.
146. Malinowska K, Neuwirt H, Cavarretta IT, Bektic J, Steiner H, Dietrich H, et al. Interleukin-6 stimulation of growth of prostate cancer in vitro and in vivo through activation of the androgen receptor. Endocrine-Related Cancer. 2009;16(1):155-69.
147. Kim HJ, Lee WJ. Ligand-independent activation of the androgen receptor by insulin-like growth factor-I and the role of the MAPK pathway in skeletal muscle cells. Molecules and Cells. 2009;28(6):589-93.

148. Lorin T, Salzburger W, Böhne A. Evolutionary fate of the androgen receptor−signaling pathway in ray-finned fishes with a special focus on cichlids. G3: Genes, Genomes, Genetics. 2015;5(11):2275-83.
149. O'Hara L, Smith LB. Androgen receptor roles in spermatogenesis and infertility. Best Practice & Research Clinical Endocrinology & Metabolism. 2015;29(4):595-605.
150. De Leo V, La Marca A, Lanzetta D, Cariello P, D'antona D, Morgante G. Effects of flutamide on pituitary and adrenal responsiveness to corticotrophin releasing factor (CRF). Clinical Endocrinology. 1998;49(1):85-9.
151. Charmandari E, Calis KA, Keil MF, Mohassel MR, Remaley A, Merke DP. Flutamide decreases cortisol clearance in patients with congenital adrenal hyperplasia. The Journal of Clinical Endocrinology & Metabolism. 2002;87(7):3197-200.
152. Goldspiel BR, Kohler DR. Flutamide: an antiandrogen for advanced prostate cancer. Journal of Endocrinology. 1990;24(6):616-23.
153. Ba ZF, Wang P, Koo DJ, Zhou M, Cioffi WG, Bland KI, et al. Testosterone receptor blockade after trauma and hemorrhage attenuates depressed adrenal function. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology. 2000;279(5):R1841-R8.
154. Kero J, Poutanen M, Zhang F-P, Rahman N, McNicol AM, Nilson JH, et al. Elevated luteinizing hormone induces expression of its receptor and promotes steroidogenesis in the adrenal cortex. Journal of Clinical Investigation. 2000;105(5):633.
155. Shire J. Degeneration of the adrenal X-zone in Tfm mice with inherited insensitivity to androgens. Journal of Endocrinology. 1976;71(3):445-6.
156. Hashmi A, Hanif F, Hanif SM, Abdullah FE, Shamim MS. Complete androgen insensitivity syndrome. J Coll Physicians Surg Pak. 2008;18(7):442-4.
157. Mason AJ, Hayflick JS, Zoeller RT, Young WS, Phillips HS, Nikolics K, et al. A deletion truncating the gonadotropin-releasing hormone gene is responsible for hypogonadism in the hpg mouse. Science. 1986;234(4782):1366-71.
158. Risbridger G, Robertson D, De Kretser D. The effects of chronic human chorionic gonadotropin treatment on Leydig cell function. Journal of Endocrinology. 1982;110(1):138-45.
159. Piltonen T, Koivunen R, Morin-Papunen L, Ruokonen A, Huhtaniemi I, Tapanainen JS. Ovarian and adrenal steroid production: regulatory role of LH/HCG. Human Reproduction. 2002;17(3):620-4.
160. Zhou X. Roles of Androgen receptor in male and female reproduction: Lessons from global and cell-specific androgen receptor knockout (ARKO) mice. Journal of Andrology. 2010;31(3):235-43.
161. Kleiman A, Tuckermann JP. Glucocorticoid receptor action in beneficial and side effects of steroid therapy: lessons from conditional knockout mice. Molecular and Cellular Endocrinology. 2007;275(1):98-108.
162. Miyamoto J, Matsumoto T, Shiina H, Inoue K, Takada I, Ito S, et al. The pituitary function of androgen receptor constitutes a glucocorticoid production circuit. Molecular and Cellular Biology. 2007;27(13):4807-14.
163. O'Hara L, McInnes K, Simitsidellis I, Morgan S, Atanassova N, Slowikowska-Hilczer J, et al. Autocrine androgen action is essential for Leydig cell maturation and function, and protects against late-onset Leydig cell apoptosis in both mice and men. The FASEB Journal. 2015;29(3):894-910.

164. De Gendt K, Swinnen JV, Saunders PT, Schoonjans L, Dewerchin M, Devos A, et al. A Sertoli cell-selective knockout of the androgen receptor causes spermatogenic arrest in meiosis. Proceedings of the National Academy of Sciences. 2004;101(5):1327-32.
165. Welsh M, Saunders PT, Atanassova N, Sharpe RM, Smith LB. Androgen action via testicular peritubular myoid cells is essential for male fertility. The FASEB Journal. 2009;23(12):4218-30.
166. Hollenberg SM, Weinberger C, Ong ES, Cerelli G, Oro A, Lebo R, et al. Primary structure and expression of a functional human glucocorticoid receptor cDNA. Nature. 1985;318(6047):635-41.
167. GALON J, Franchimont D, Hiroi N, Frey G, Boettner A, Ehrhart-Bornstein M, et al. Gene profiling reveals unknown enhancing and suppressive actions of glucocorticoids on immune cells. The FASEB Journal. 2002;16(1):61-71.
168. Lu NZ, Collins JB, Grissom SF, Cidlowski JA. Selective regulation of bone cell apoptosis by translational isoforms of the glucocorticoid receptor. Molecular and Cellular Biology. 2007;27(20):7143-60.
169. Ren R, Oakley RH, Cruz-Topete D, Cidlowski JA. Dual role for glucocorticoids in cardiomyocyte hypertrophy and apoptosis. Journal of Endocrinology. 2012;153(11):5346-60.
170. Cole TJ, Blendy JA, Monaghan AP, Krieglstein K, Schmid W, Aguzzi A, et al. Targeted disruption of the glucocorticoid receptor gene blocks adrenergic chromaffin cell development and severely retards lung maturation. Genes & Development. 1995;9(13):1608-21.
171. Lu NZ, Cidlowski JA. Translational regulatory mechanisms generate N-terminal glucocorticoid receptor isoforms with unique transcriptional target genes. Molecular Cell. 2005;18(3):331-42.
172. Oakley RH, Cidlowski JA. Cellular processing of the glucocorticoid receptor gene and protein: new mechanisms for generating tissue-specific actions of glucocorticoids. Journal of Biological Chemistry. 2011;286(5):3177-84.
173. Kumar R, McEwan IJ. Glucocorticoid Receptor Structure and Function. Nuclear Receptors: From Structure to the Clinic: Springer; 2015. p. 41-57.
174. Castro-Vale I, van Rossum EF, Machado JC, Mota-Cardoso R, Carvalho D. Genetics of glucocorticoid regulation and posttraumatic stress disorder—What do we know? Neuroscience & Biobehavioral Reviews. 2016;63:143-57.
175. Bledsoe RK, Montana VG, Stanley TB, Delves CJ, Apolito CJ, McKee DD, et al. Crystal structure of the glucocorticoid receptor ligand binding domain reveals a novel mode of receptor dimerization and coactivator recognition. Cell. 2002;110(1):93-105.
176. Zhou J, Cidlowski JA. The human glucocorticoid receptor: one gene, multiple proteins and diverse responses. Steroids. 2005;70(5):407-17.
177. Pratt W. The role of heat shock proteins in regulating the function, folding, and trafficking of the glucocorticoid receptor. Journal of Biological Chemistry. 1993;267-278.
178. Pratt WB, Toft DO. Steroid receptor interactions with heat shock protein and immunophilin chaperones. Endocrine Reviews. 1997;18(3):306-60.
179. Yang H, Dou W, Lou J, Leng Y, Shen J. Discovery of novel inhibitors of 11β-hydroxysteroid dehydrogenase type 1 by docking and pharmacophore modeling. Bioorganic & Medicinal Chemistry Letters. 2008;18(4):1340-5.

180. Uhlenhaut NH, Barish GD, Ruth TY, Downes M, Karunasiri M, Liddle C, et al. Insights into negative regulation by the glucocorticoid receptor from genome-wide profiling of inflammatory cistromes. Molecular Cell. 2013;49(1):158-71.
181. Surjit M, Ganti KP, Mukherji A, Ye T, Hua G, Metzger D, et al. Widespread negative response elements mediate direct repression by agonist-liganded glucocorticoid receptor. Cell. 2011;145(2):224-41.
182. Ronacher K, Hadley K, Avenant C, Stubsrud E, Simons SS, Louw A, et al. Ligand-selective transactivation and transrepression via the glucocorticoid receptor: role of cofactor interaction. Molecular and Cellular Endocrinology. 2009;299(2):219-31.
183. Tanenbaum DM, Wang Y, Williams SP, Sigler PB. Crystallographic comparison of the estrogen and progesterone receptor's ligand binding domains. Proceedings of the National Academy of Sciences. 1998;95(11):5998-6003.
184. Auboeuf D, Hönig A, Berget SM, O'malley BW. Coordinate regulation of transcription and splicing by steroid receptor coregulators. Science. 2002;298(5592):416-9.
185. Göttlicher M, Heck S, Herrlich P. Transcriptional cross-talk, the second mode of steroid hormone receptor action. Journal of Molecular Medicine. 1998;76(7):480-9.
186. Solito E, Mulla A, Morris JF, Christian HC, Flower RJ, Buckingham JC. Dexamethasone induces rapid serine-phosphorylation and membrane translocation of annexin 1 in a human folliculostellate cell line via a novel nongenomic mechanism involving the glucocorticoid receptor, protein kinase C, phosphatidylinositol 3-kinase, and mitogen-activated protein kinase. Journal of Endocrinology. 2003;144(4):1164-74.
187. Croxtall JD, Choudhury Q, Flower RJ. Glucocorticoids act within minutes to inhibit recruitment of signalling factors to activated EGF receptors through a receptor-dependent, transcription-independent mechanism. British Journal of Pharmacology. 2000;130(2):289-98.
188. Matthews L, Berry A, Ohanian V, Ohanian J, Garside H, Ray D. Caveolin mediates rapid glucocorticoid effects and couples glucocorticoid action to the antiproliferative program. Molecular Endocrinology. 2008;22(6):1320-30.
189. Samarasinghe RA, Di Maio R, Volonte D, Galbiati F, Lewis M, Romero G, et al. Nongenomic glucocorticoid receptor action regulates gap junction intercellular communication and neural progenitor cell proliferation. Proceedings of the National Academy of Sciences. 2011;108(40):16657-62.
190. Martinerie L, Munier M, Le Menuet D, Meduri G, Viengchareun S, Lombès M. The mineralocorticoid signaling pathway throughout development: expression, regulation and pathophysiological implications. Biochimie. 2013;95(2):148-57.
191. Woods C, Tomlinson JW. The dehydrogenase hypothesis. Glucocorticoid Signaling: Springer; 2015. p. 353-80.
192. Kalra N, Ishmael F. Cross-talk between vitamin D, estrogen and corticosteroids in glucocorticoid resistant asthma. OA inflammation. 2014;2:2-10.
193. Ridder S, Chourbaji S, Hellweg R, Urani A, Zacher C, Schmid W, et al. Mice with genetically altered glucocorticoid receptor expression show altered sensitivity for stress-induced depressive reactions. Journal of Neuroscience. 2005;25(26):6243-50.

194. Brewer JA, Kanagawa O, Sleckman BP, Muglia LJ. Thymocyte apoptosis induced by T cell activation is mediated by glucocorticoids in vivo. The Journal of Immunology. 2002;169(4):1837-43.
195. Tronche F, Kellendonk C, Kretz O, Gass P, Anlag K, Orban PC, et al. Disruption of the glucocorticoid receptor gene in the nervous system results in reduced anxiety. Nature. 1999;23(1):99-103.
196. Reichardt HM, Kaestner KH, Tuckermann J, Kretz O, Wessely O, Bock R, et al. DNA binding of the glucocorticoid receptor is not essential for survival. Cell. 1998;93(4):531-41.
197. Boyle MP, Brewer JA, Funatsu M, Wozniak DF, Tsien JZ, Izumi Y, et al. Acquired deficit of forebrain glucocorticoid receptor produces depression-like changes in adrenal axis regulation and behavior. Proceedings of the National Academy of Sciences. 2005;102(2):473-8.
198. Reichardt HM, Umland T, Bauer A, Kretz O, Schütz G. Mice with an increased glucocorticoid receptor gene dosage show enhanced resistance to stress and endotoxic shock. Molecular and Cellular Biology. 2000;20(23):9009-17.
199. Wei Q, Lu X-Y, Liu L, Schafer G, Shieh K-R, Burke S, et al. Glucocorticoid receptor overexpression in forebrain: a mouse model of increased emotional lability. Proceedings of the National Academy of Sciences. 2004;101(32):11851-6.
200. Claessens F, Joniau S, Helsen C. Comparing the rules of engagement of androgen and glucocorticoid receptors. Cellular and Molecular Life Sciences. 2017:1-12.
201. Gregory CW, He B, Johnson RT, Ford OH, Mohler JL, French FS, et al. A mechanism for androgen receptor-mediated prostate cancer recurrence after androgen deprivation therapy. Cancer Research. 2001;61(11):4315-9.
202. Kach J, Conzen SD, Szmulewitz RZ. Glucocorticoid receptor signaling in breast and prostate cancers: emergence as a therapeutic target. Science Translational Medicine. 2015;7(305):305ps19.
203. Xie N, Cheng H, Lin D, Liu L, Yang O, Jia L, et al. The expression of glucocorticoid receptor is negatively regulated by active androgen receptor signaling in prostate tumors. International Journal of Cancer. 2015;60(4):136-145.
204. McBeth L, Grabnar M, Selman S, Hinds TD. Involvement of the androgen and glucocorticoid receptors in bladder cancer. International Journal of Endocrinology. 2015;2015.
205. Chen S-y, Wang J, Yu G-q, Liu W, Pearce D. Androgen and Glucocorticoid Receptor Heterodimer Formation. A possible mechanism for mutual inhibition of transcriptional activity. Journal of Biological Chemistry. 1997;272(22):14087-92.
206. Chrousos G. The neuroendocrinology of stress: Its relation to the hormonal milieu, growth, and development. Growth Genet Horm. 1997;13:1-8.
207. Habib KE, Gold PW, Chrousos GP. Neuroendocrinology of stress. Endocrinology and Metabolism Clinics. 2001;30(3):695-728.
208. Clow A, Hucklebridge F, Stalder T, Evans P, Thorn L. The cortisol awakening response: more than a measure of HPA axis function. Neuroscience & Biobehavioral Reviews. 2010;35(1):97-103.
209. Rivier C, Vale W. Modulation of stress-induced ACTH release by corticotropin-releasing factor, catecholamines and vasopressin. Nature. 1983;305(5932):325-7.

210. Majzoub. Corticotropin-releasing hormone physiology. Journal of Endocrinology. 2006;155(suppl 1):S71-S6.
211. Perrin M, Donaldson C, Chen R, Blount A, Berggren T, Bilezikjian L, et al. Identification of a second corticotropin-releasing factor receptor gene and characterization of a cDNA expressed in heart. Proceedings of the National Academy of Sciences. 1995;92(7):2969-73.
212. Van Pett K, Viau V, Bittencourt JC, Chan RK, Li HY, Arias C, et al. Distribution of mRNAs encoding CRF receptors in brain and pituitary of rat and mouse. Journal of Comparative Neurology. 2000;428(2):191-212.
213. Brownstein MJ, Russell JT, Gainer H. Synthesis, transport, and release of posterior pituitary hormones. Science. 1980;207(4429):373-8.
214. Birnbaumer M. Vasopressin receptors. Trends in Endocrinology & Metabolism. 2000;11(10):406-10.
215. Chang A, Cochet M, Cohen SN. Structural organization of human genomic DNA encoding the pro-opiomelanocortin peptide. Proceedings of the National Academy of Sciences. 1980;77(8):4890-4.
216. Mountjoy KG, Robbins LS, Mortrud MT, Cone RD. The cloning of a family of genes that encode the melanocortin receptors. Science. 1992;257(5074):1248-51.
217. Raffin-Sanson M, De Keyzer Y, Bertagna X. Proopiomelanocortin, a polypeptide precursor with multiple functions: from physiology to pathological conditions. Journal of Endocrinology. 2003;149(2):79-90.
218. Sawchenko P. Evidence for a local site of action for glucocorticoids in inhibiting CRF and vasopressin expression in the paraventricular nucleus. Brain Research. 1987;403(2):213-24.
219. Reul J, Kloet Ed. Two receptor systems for corticosterone in rat brain: microdistribution and differential occupation. Journal of Endocrinology. 1985;117(6):2505-11.
220. Briassoulis G, Damjanovic S, Xekouki P, Lefebvre H, Stratakis C. The glucocorticoid receptor and its expression in the anterior pituitary and the adrenal cortex: a source of variation in hypothalamic-pituitary-adrenal axis function; Implications for pituitary and andrenal tumors. Journal of Reproductive Endocrinology. 2011;17(6):941-8.
221. Tasker JG, Herman JP. Mechanisms of rapid glucocorticoid feedback inhibition of the hypothalamic–pituitary–adrenal axis. Stress. 2011;14(4):398-406.
222. Hinz B, Hirschelmann R. Rapid non-genomic feedback effects of glucocorticoids on CRF-induced ACTH secretion in rats. Pharmaceutical Research. 2000;17(10):1273-7.
223. Chrousos GP, Gold PW. The concepts of stress and stress system disorders: overview of physical and behavioral homeostasis. JAMA. 1992;267(9):1244-52.
224. Ulrich-Lai YM, Figueiredo HF, Ostrander MM, Choi DC, Engeland WC, Herman JP. Chronic stress induces adrenal hyperplasia and hypertrophy in a subregion-specific manner. American Journal of Physiology-Endocrinology and Metabolism. 2006;291(5):E965-E73.
225. Plant TM. 60 Years of Neuroendocrinology: The hypothalamo-pituitary–gonadal axis. Journal of Endocrinology. 2015;226(2):T41-T54.
226. Rivier C, Rivest S. Effect of stress on the activity of the hypothalamic-pituitary-gonadal axis: peripheral and central mechanisms. Biology of Reproduction. 1991;45(4):523-32.

227. Tilbrook A, Turner A, Clarke I. Effects of stress on reproduction in non-rodent mammals: the role of glucocorticoids and sex differences. Reviews of Reproduction. 2000;5(2):105-13.
228. Dallman MF, Viau VG, Bhatnagar S, Gomez F, Laugero K, Bell M. Corticotropin-releasing factor, corticosteroids, stress, and sugar: energy balance, the brain, and behavior. Hormones, Brain and Behavior. 2002;1:571-631.
229. Selye H. Stress without distress. Psychopathology of Human Adaptation. New York. 1974:26-39.
230. Young EA, Haskett RF, Murphy-Weinberg V, Watson SJ, Akil H. Loss of glucocorticoid fast feedback in depression. Archives of General Psychiatry. 1991;48(8):693-9.
231. Holsboer F. The corticosteroid receptor hypothesis of depression. Neuropsychopharmacology. 2000;23(5):477-501.
232. Anacker C, Zunszain PA, Carvalho LA, Pariante CM. The glucocorticoid receptor: pivot of depression and of antidepressant treatment? Psychoneuroendocrinology. 2011;36(3):415-25.
233. Ehlert U, Gaab J, Heinrichs M. Psychoneuroendocrinological contributions to the etiology of depression, posttraumatic stress disorder, and stress-related bodily disorders: the role of the hypothalamus–pituitary–adrenal axis. Biological Psychology. 2001;57(1):141-52.
234. Barden N. Implication of the hypothalamic–pituitary–adrenal axis in the physiopathology of depression. Journal of Psychiatry and Neuroscience. 2004;29(3):185-192.
235. Daubert DL, Looney BM, Clifton RR, Cho JN, Scheuer DA. Elevated corticosterone in the dorsal hindbrain increases plasma norepinephrine and neuropeptide Y, and recruits a vasopressin response to stress. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology. 2014;307(2):R212-R24.
236. Barden N, Beaulieu S. Anti-depressant, serotoninergic and glucocorticoid modulation of glucocorticoid receptor messenger-RNA in hypothalamic neurons. Endocrinology Letters. 1987; 9(3):186-186.
237. Dedic N. Genetic dissection of CRH-controlled neurocircuitries of stress. The Neuropsychology of Anxiety. California. 2015:13-20.
238. Chen L-S, Eaton WW, Gallo JJ, Nestadt G. Understanding the heterogeneity of depression through the triad of symptoms, course and risk factors: a longitudinal, population-based study. Journal of Affective Disorders. 2000;59(1):1-11.
239. Schmidt M, Deussing J, Oitzl M, Ohl F, Levine S, Wurst W, et al. Differential disinhibition of the neonatal hypothalamic–pituitary–adrenal axis in brain-specific CRH receptor 1-knockout mice. European Journal of Neuroscience. 2006;24(8):2291-8.
240. Earls F. Sex differences in psychiatric disorders: origins and developmental influences. Psychiatric Developments. 1987;5(1):1-23.
241. Granger DA, Shirtcliff EA, Zahn–Waxler C, Usher B, Klimes–Dougan B, Hastings P. Salivary testosterone diurnal variation and psychopathology in adolescent males and females: Individual differences and developmental effects. Development and Psychopathology. 2003;15(2):431-49.
242. Davis S. Testosterone deficiency in women. The Journal of Reproductive Medicine. 2001;46(3 Suppl):291-6.

243. Osborne DM, Edinger K, Frye CA. Chronic administration of androgens with actions at estrogen receptor beta have anti-anxiety and cognitive-enhancing effects in male rats. Age. 2009;31(3):191-8.
244. Frye CA, Edinger K, Sumida K. Androgen administration to aged male mice increases anti-anxiety behavior and enhances cognitive performance. Neuropsychopharmacology: 2008;33(5):1049.
245. Chen CV, Brummet JL, Jordan CL, Breedlove SM. Down, but not out: partial elimination of androgen receptors in the male mouse brain does not affect androgenic regulation of anxiety or HPA activity.Journal of Endocrinology. 2015;157(2):764-73.
246. Howard-Miller E. A transitory zone in the adrenal cortex which shows age and sex relationships. Developmental Dynamics. 1927;40(2):251-93.
247. Yeh S, Tsai M-Y, Xu Q, Mu X-M, Lardy H, Huang K-E, et al. Generation and characterization of androgen receptor knockout (ARKO) mice: an in vivo model for the study of androgen functions in selective tissues. Proceedings of the National Academy of Sciences. 2002;99(21):13498-503.
248. Zhang R, Jankord R, Flak JN, Solomon MB, D'Alessio DA, Herman JP. Role of glucocorticoids in tuning hindbrain stress integration. Journal of Neuroscience. 2010;30(44):14907-14.
249. Sapolsky RM, Meaney MJ. Maturation of the adrenocortical stress response: neuroendocrine control mechanisms and the stress hyporesponsive period. Brain Research Reviews. 1986;11(1):65-76.
250. Bechtold AG, Vernon K, Scheuer DA. Effects of chronic dorsal hindbrain glucocorticoid receptor blockade on cardiovascular responses to acute stress. The FASEB Journal. 2006;20(4):A771.
251. Truica CI, Byers S, Gelmann EP. β-Catenin affects androgen receptor transcriptional activity and ligand specificity. Cancer research. 2000;60(17):4709-13.
252. Arora VK, Schenkein E, Murali R, Subudhi SK, Wongvipat J, Balbas MD, et al. Glucocorticoid receptor confers resistance to antiandrogens by bypassing androgen receptor blockade. Cell. 2013;155(6):1309-22.
253. O'Hara L, York JP, Zhang P, Smith LB. Targeting of GFP-Cre to the mouse Cyp11a1 locus both drives cre recombinase expression in steroidogenic cells and permits generation of Cyp11a1 knock out mice. PloS one. 2014 Jan 3;9(1):e84541.
254. Kilkenny C, Browne W, Cuthill IC, Emerson M, Altman DG. Animal research: reporting in vivo experiments: the ARRIVE guidelines. British Journal of Pharmacology. 2010;160(7):1577-9.
255. Nagy A. Cre recombinase: the universal reagent for genome tailoring. Genesis. 2000;26(2):99.
256. Wang Y, Yau Y-Y, Perkins-Balding D, Thomson JG. Recombinase technology: applications and possibilities. Plant cell reports. 2011;30(3):267-85.
257. Garcia EL, Mills AA, editors. Getting around lethality with inducible Cre-mediated excision. Seminars in cell & developmental biology; 2002: Academic Press.
258. Pechisker A. Targeting your DNA with the Cre/*loxP* system [Textbook]. 2004. Available from: http://www.scq.ubc.ca/targeting-your-dna-with-the-crelox-system/.
259. Friedrich G, Soriano P. Promoter traps in embryonic stem cells: a genetic screen to identify and mutate developmental genes in mice. Genes & Development. 1991;5(9):1513-23.

260. Srinivas S, Watanabe T, Lin C-S, William CM, Tanabe Y, Jessell TM, et al. Cre reporter strains produced by targeted insertion of EYFP and ECFP into the ROSA26 locus. Journal of Developmental Biology. 2001;1(1):4-11.
261. Hellwinkel OJ-C, Bull K, Holterhus P-M, Homburg N, Struve D, Hiort O. Complete androgen insensitivity caused by a splice donor site mutation in intron 2 of the human androgen receptor gene resulting in an exon 2-lacking transcript with premature stop-codon and reduced expression. The Journal of Steroid Biochemistry and Molecular Biology. 1999;68(1):1-9.
262. Welsh M, Saunders PT, Fisken M, Scott HM, Hutchison GR, Smith LB, et al. Identification in rats of a programming window for reproductive tract masculinization, disruption of which leads to hypospadias and cryptorchidism. The Journal of Clinical Investigation. 2008;118(4):1479.
263. Kalueff A, Minasyan A, Keisala T, Shah Z, Tuohimaa P. Hair barbering in mice: implications for neurobehavioural research. Behavioural Processes. 2006;71(1):8-15.
264. Garner JP, Dufour B, Gregg LE, Weisker SM, Mench JA. Social and husbandry factors affecting the prevalence and severity of barbering ('whisker trimming') by laboratory mice. Applied Animal Behaviour Science. 2004;89(3):263-82.
265. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative CT method. Nature protocols. 2008;3(6):1101-1110.
266. COBB JR. Outline for the study of scoliosis. Instructional Course Lectures. 1948.
267. Pochaczevsky R, Wexler CE, Meyers PH, Epstein JA, Marc JA. Liquid crystal thermography of the spine and extremities: Its value in the diagnosis of spinal root syndromes. Journal of Neurosurgery. 1982;56(3):386-95.
268. Laughlin GA, Barrett-Connor E, Bergstrom J. Low serum testosterone and mortality in older men. The Journal of Clinical Endocrinology & Metabolism. 2008;93(1):68-75.
269. Murashima A, Kishigami S, Thomson A, Yamada G. Androgens and mammalian male reproductive tract development. Biochimica et Biophysica Acta (BBA)-Gene Regulatory Mechanisms. 2015 Feb 28;1849(2):163-70.
270. Isidori AM, Caprio M, Strollo F, Moretti C, Frajese G, Isidori A, et al. Leptin and androgens in male obesity: Evidence for leptin contribution to reduced androgen levels 1. The Journal of Clinical Endocrinology & Metabolism. 1999;84(10):3673-80.
271. Kupelian V, Hayes FJ, Link CL, Rosen R, McKinlay JB. Inverse association of testosterone and the metabolic syndrome in men is consistent across race and ethnic groups. The Journal of Clinical Endocrinology & Metabolism. 2008;93(9):3403-10.
272. Gottlieb B, Lombroso R, Beitel LK, Trifiro MA. Molecular pathology of the androgen receptor in male (in) fertility. Reproductive Biomedicine. 2005;10(1):42-8.
273. Foradori C, Weiser M, Handa R. Non-genomic actions of androgens. Frontiers in neuroendocrinology. 2008;29(2):169-81.
274. Franchimont P. Regulation of gonadal androgen secretion. Hormone Research in Paediatrics. 1983;18(1-3):7-17.
275. Bentvelsen F, McPhaul M, Wilson C, Wilson J, George F. Regulation of immunoreactive androgen receptor in the adrenal gland of the adult rat. Journal of Endocrinology. 1996;137(7):2659-63.

276. Sar M, Lubahn DB, French FS, Wilson EM. Immunohistochemical localization of the androgen receptor in rat and human tissues. Journal of Endocrinology. 1990;127(6):3180-6.
277. Lerario AM, Finco I, LaPensee C, Hammer GD. Molecular mechanisms of stem/progenitor cell maintenance in the adrenal cortex. Frontiers in Endocrinology. 2017;8.
278. Lanman JT. The fetal zone of the adrenal gland: its developmental course, comparative anatomy, and possible physiologic functions. Medicine. 1953;32(4):389-430.
279. Rossi R, Zatelli M, Valentini A, Cavazzini P, Fallo F, Del Senno L, et al. Evidence for androgen receptor gene expression and growth inhibitory effect of dihydrotestosterone on human adrenocortical cells. Journal of Endocrinology. 1998;159(3):373-80.
280. Rosol TJ, Yarrington JT, Latendresse J, Capen CC. Adrenal gland: structure, function, and mechanisms of toxicity. Toxicologic pathology. 2001;29(1):41-8.
281. Hershkovitz L, Beuschlein F, Klammer S, Krup M, Weinstein Y. Adrenal 20α-hydroxysteroid dehydrogenase in the mouse catabolizes progesterone and 11-deoxycorticosterone and is restricted to the X-zone. Journal of Endocrinology. 2007;148(3):976-88.
282. Gong S, Miao Y-L, Jiao G-Z, Sun M-J, Li H, Lin J, et al. Dynamics and correlation of serum cortisol and corticosterone under different physiological or stressful conditions in mice. PloS One. 2015;10(2):e0117503.
283. Belgardt BF, Husch A, Rother E, Ernst MB, Wunderlich FT, Hampel B, et al. PDK1 deficiency in POMC-expressing cells reveals FOXO1-dependent and-independent pathways in control of energy homeostasis and stress response. Cell Metabolism. 2008;7(4):291-301.
284. Rubin BL, Deane HW, Hamilton JA. Biochemical and histochemical studies on Δ5-3β-hydroxysteroid dehydrogenase activity in the adrenal glands and ovaries of diverse mammals. Journal of Endocrinology. 1963;73(6):748-63.
285. Drelon C, Berthon A, Sahut-Barnola I, Mathieu M, Dumontet T, Rodriguez S, et al. PKA inhibits WNT signalling in adrenal cortex zonation and prevents malignant tumour development. Nature. 2016;7.
286. De Kloet ER, DeRijk RH, Meijer OC. Therapy insight: is there an imbalanced response of mineralocorticoid and glucocorticoid receptors in depression? Nature Reviews Endocrinology. 2007;3(2):168-177.
287. Carney JA. Unusual tumefactive spindle-cell lesions in the adrenal glands. Human Pathology. 1987;18(10):980-5.
288. Stanbrough M, Bubley GJ, Ross K, Golub TR, Rubin MA, Penning TM, et al. Increased expression of genes converting adrenal androgens to testosterone in androgen-independent prostate cancer. Cancer Research. 2006;66(5):2815-25.
289. Sgoifo A, De Boer SF, Haller J, Koolhaas JM. Individual differences in plasma catecholamine and corticosterone stress responses of wild-type rats: relationship with aggression. Physiology & Behavior. 1996;60(6):1403-7.
290. Gross DS. Effect of castration and steroid replacement on immunoreactive gonadotropin-releasing hormone in the hypothalamus and preoptic area. Journal of Endocrinology. 1980;106(5):1442-50.
291. Drouin J, Fernand L. Selective effect of androgens on LH and FSH release in anterior pituitary cells in culture. Journal of Endocrinology. 1976;98(6):1528-34.

292. Malisch JL, Saltzman W, Gomes FR, Rezende EL, Jeske DR, Garland Jr T. Baseline and stress-induced plasma corticosterone concentrations of mice selectively bred for high voluntary wheel running. Physiological and Biochemical Zoology. 2006;80(1):146-56.
293. Val P, Lefrançois-Martinez A-M, Veyssière G, Martinez A. SF-1 a key player in the development and differentiation of steroidogenic tissues. Nuclear Receptor. 2003;1(1):8.
294. Teets BW, Soprano KJ, Soprano DR. Role of SF-1 and DAX-1 during differentiation of P19 cells by retinoic acid. Journal of Cellular Physiology. 2012;227(4):1501-11.
295. Wood MA, Hammer GD. Adrenocortical stem and progenitor cells: unifying model of two proposed origins. Molecular and Cellular Endocrinology. 2011;336(1):206-12.
296. Sahut-Barnola I, Lefrancois-Martinez A, Jean C, Veyssiere G, Martinez A. Adrenal tumorigenesis targeted by the corticotropin-regulated promoter of the aldo-keto reductase AKR1B7 gene in transgenic mice. Endocrine Research. 2000;26(4):885-98.
297. Martinez A, Aigueperse C, Val P, Dussault M-H, Tournaire C, Berger M, et al. Physiological functions and hormonal regulation of mouse vas deferens protein (AKR1B7) in steroidogenic tissues. Chemico-Biological Interactions. 2001;130:903-17.
298. Martinez A, Val P, Sahut-Barnola I, Aigueperse C, Veyssière G, Lefrançois-Martinez A-M. Steroidogenic factor-1 controls the aldose reductase akr1b7 gene promoter in transgenic mice through an atypical binding site. Journal of Endocrinology. 2003;144(5):2111-20.
299. Kim J-S, Kubota H, Doi K, Saegusa J. Correlation of mast cells with spindle cell hyperplasia in the adrenal cortex of IQI/Jic mice. Experimental Animals. 1997;46(2):103-9.
300. Ross E, Marshall-Jones P, Friedman M. Cushing's syndrome: diagnostic criteria. QJM. 1966;35(2):149-92.
301. Whitworth JA, Williamson PM, Mangos G, Kelly JJ. Cardiovascular consequences of cortisol excess. Vascular health and risk management. 2005;1(4):291.
302. Freehill AK, Lenke LG. Severe kyphosis secondary to glucocorticoid-induced osteoporosis in a young adult with cushing's disease: a case report and literature review. Spine. 1999;24(2):189-93.
303. Chang C, Chen Y-T, Yeh S-D, Xu Q, Wang R-S, Guillou F, et al. Infertility with defective spermatogenesis and hypotestosteronemia in male mice lacking the androgen receptor in Sertoli cells. Proceedings of the National Academy of Sciences. 2004;101(18):6876-81.
304. McGinnis MY, Williams GW, Lumia AR. Inhibition of male sex behavior by androgen receptor blockade in preoptic area or hypothalamus, but not amygdala or septum. Physiology & Behavior. 1996;60(3):783-9.
305. Wang R-S, Yeh S, Tzeng C-R, Chang C. Androgen receptor roles in spermatogenesis and fertility: lessons from testicular cell-specific androgen receptor knockout mice. Endocrine Reviews. 2009;30(2):119-32.
306. Hu Y-C, Wang P-H, Yeh S, Wang R-S, Xie C, Xu Q, et al. Subfertility and defective folliculogenesis in female mice lacking androgen receptor. Proceedings of the National Academy of Sciences. 2004;101(31):11209-14.

307. Bai S, Grossman G, Yuan L, Lessey BA, French FS, Young SL, et al. Hormone control and expression of androgen receptor coregulator MAGE-11 in human endometrium during the window of receptivity to embryo implantation. MHR-Basic Science of Reproductive Medicine. 2007;14(2):107-16.
308. Hickey T, Robinson J, Carroll J, Tilley W. Minireview: the androgen receptor in breast tissues: growth inhibitor, tumor suppressor, oncogene? Journal of Molecular Endocrinology. 2012;26(8):1252-67.
309. Burger HG. Androgen production in women. Fertility and Sterility. 2002;77:3-5.
310. Parker Jr CR, Slayden SM, Azziz R, Crabbe SL, Hines GA, Boots LR, et al. Effects of aging on adrenal function in the human: responsiveness and sensitivity of adrenal androgens and cortisol to adrenocorticotropin in premenopausal and postmenopausal women. The Journal of Clinical Endocrinology & Metabolism. 2000;85(1):48-54.
311. Waring H. Effect of hormones on degeneration of the x-zone in the mouse adrenal. Journal of Endocrinology. 1942;3(1):123-31.
312. Libé R, Bertherat J. Molecular genetics of adrenocortical tumours, from familial to sporadic diseases. Journal of Endocrinology. 2005;153(4):477-87.
313. Palter V, Devon K, Hallet J, Kulkarni GS, Law CH, Urbach DR. Tumours of the Adrenal Gland. Surgical Oncology Manual: Springer; 2016. p. 1-12.
314. Kim J-S, Kubota H, Kiuchi Y, Doi K, Saegusa J. Subcapsular cell hyperplasia and mast cell infiltration in the adrenal cortex of mice: comparative study in 7 inbred strains. Experimental Animals. 1997;46(4):303-6.
315. Suzuki DT, Griffiths AJ. An introduction to genetic analysis: WH Freeman and Company.; 1976.
316. Spinedi E, Gaillard RC. A regulatory loop between the hypothalamo-pituitary-adrenal (HPA) axis and circulating leptin: a physiological role of ACTH. Journal of Endocrinology. 1998;139(9):4016-20.
317. Mitrovic. Introduction to the hypothalamopituitary-adrenal (HPA) axis. Biochemistry. 2002:465-86.
318. Meikle A, Daynes R, Araneo B. Adrenal androgen secretion and biologic effects. Endocrinology and Metabolism Clinics of North America. 1991;20(2):381-400.
319. Arif S, Vallian S, Farzaneh F, Zanone MM, James SL, Pietropaolo M, et al. Identification of 3 beta-hydroxysteroid dehydrogenase as a novel target of steroid cell autoantibodies: association of autoantibodies with endocrine autoimmune disease. The Journal of Clinical Endocrinology & Metabolism. 1996;81(12):4439-45.
320. Tacon LJ, Soon PS, Gill AJ, Chou AS, Clarkson A, Botling J, et al. The glucocorticoid receptor is overexpressed in malignant adrenocortical tumors. The Journal of Clinical Endocrinology & Metabolism. 2009;94(11):4591-9.
321. Dietrich DR. Toxicological and pathological applications of proliferating cell nuclear antigen (PCNA), a novel endogenous marker for cell proliferation. Critical Reviews in Toxicology. 1993;23(1):77-109.
322. Elmore S. Apoptosis: a review of programmed cell death. Toxicologic Pathology. 2007;35(4):495-516.
323. Pihlajoki. Adrenocortical zonation, renewal, and remodeling. Frontiers in Endocrinology. 2015;6-11.

324. Lebduška P, Korb J, Tůmová M, Heneberg P, Dráber P. Topography of signaling molecules as detected by electron microscopy on plasma membrane sheets isolated from non-adherent mast cells. Journal of Immunological Methods. 2007;328(1):139-51.
325. Atkinson HC, Waddell BJ. Circadian variation in basal plasma corticosterone and adrenocorticotropin in the rat: sexual dimorphism and changes across the estrous cycle. Journal of Endocrinology. 1997;138(9):3842-8.
326. Lefrançois-Martinez A-M, Tournaire C, Martinez A, Berger M, Daoudal S, Tritsch D, et al. Product of side-chain cleavage of cholesterol, isocaproaldehyde, is an endogenous specific substrate of mouse vas deferens protein, an aldose reductase-like protein in adrenocortical cells. Journal of Biological Chemistry. 1999;274(46):32875-80.
327. Brummelte S, Pawluski JL, Galea LA. High post-partum levels of corticosterone given to dams influence postnatal hippocampal cell proliferation and behavior of offspring: a model of post-partum stress and possible depression. Hormones and Behavior. 2006;50(3):370-82.
328. Clipstone NA, Crabtree GR. Identification of calcineurin as a key signalling enzyme in T-lymphocyte activation. Nature. 1992;357(6380):695-701.
329. Vegiopoulos A, Herzig S. Glucocorticoids, metabolism and metabolic diseases. Molecular and Cellular Endocrinology. 2007;275(1):43-61.
330. Smoak KA, Cidlowski JA. Mechanisms of glucocorticoid receptor signaling during inflammation. Mechanisms of Ageing and Development. 2004;125(10):697-706.
331. Perkins ND. Integrating cell-signalling pathways with NF-[kappa] B and IKK function. Nature reviews Molecular Cell Biology. 2007;8(1):49-55.
332. Belzung C, Griebel G. Measuring normal and pathological anxiety-like behaviour in mice: a review. Behavioural Brain Research. 2001;125(1):141-9.
333. Loose DS, Do YS, Chen TL, Feldman D. Demonstration of glucocorticoid receptors in the adrenal cortex: evidence for a direct dexamethasone suppressive effect on the rat adrenal gland. Journal of Endocrinology. 1980;107(1):137-46.
334. Paust H-J, Loeper S, Else T, Bamberger A-M, Papadopoulos G, Pankoke D, et al. Expression of the glucocorticoid receptor in the human adrenal cortex. Experimental and Clinical Endocrinology & Diabetes. 2006;114(01):6-10.
335. Condon J, Gosden C, Gardener D, Nickson P, Hewison M, Howie AJ, et al. Expression of type 2 11β-hydroxysteroid dehydrogenase and corticosteroid hormone receptors in early human fetal life. The Journal of Clinical Endocrinology & Metabolism. 1998;83(12):4490-7.
336. Bourdeau I, Lacroix A, Schürch W, Caron P, Antakly T, Stratakis CA. Primary pigmented nodular adrenocortical disease: paradoxical responses of cortisol secretion to dexamethasone occur in vitro and are associated with increased expression of the glucocorticoid receptor. The Journal of Clinical Endocrinology & Metabolism. 2003;88(8):3931-7.
337. Hartig SM, He B, Newberg JY, Ochsner SA, Loose DS, Lanz RB, et al. Feed-forward inhibition of androgen receptor activity by glucocorticoid action in human adipocytes. Chemistry & Biology. 2012;19(9):1126-41.
338. Balbas MD, Evans MJ, Hosfield DJ, Wongvipat J, Arora VK, Watson PA, et al. Overcoming mutation-based resistance to antiandrogens with rational drug design. Elife. 2013;2:e00499.

339. Urani A, Chourbaji S, Gass P. Mutant mouse models of depression: candidate genes and current mouse lines. Neuroscience & Biobehavioral Reviews. 2005;29(4):805-28.
340. Bailey KR, Crawley JN. Anxiety-related behaviors in mice. 2009. Chapter 5.
341. Denmark A, Tien D, Wong K, Chung A, Cachat J, Goodspeed J, et al. The effects of chronic social defeat stress on mouse self-grooming behavior and its patterning. Behavioural Brain Research. 2010;208(2):553-9.
342. Lee SW, Hong JT, Son BC, Sung JH, Kim IS, Park CK. Analysis of accuracy of kyphotic angle measurement for vertebral osteoporotic compression fractures. Journal of Clinical Neuroscience. 2007;14(10):961-5.
343. Rodgers R, Cole J. Influence of social isolation, gender, strain, and prior novelty on plus-maze behaviour in mice. Physiology & behavior. 1993;54(4):729-36.
344. UK WCCR. Hindbrain schematic. Wikipedia CommonsFeb. 25, 2009.
345. Ulrich-Lai YM, Herman JP. Neural regulation of endocrine and autonomic stress responses. Nature Reviews Neuroscience. 2009;10(6):397-409.
346. Juruena MF, Cleare AJ, Pariante CM. The hypothalamic pituitary adrenal axis, glucocorticoid receptor function and relevance to depression. Revista Brasileira de Psiquiatria. 2004;26(3):189-201.
347. Dedic N, Walser SM, Deussing JM. Mouse models of depression. Psychiatric Disorders-Trends and Developments: InTech; 2011.
348. Webster M, Knable M, O'grady J, Orthmann J, Weickert CS. Regional specificity of brain glucocorticoid receptor mRNA alterations in subjects with schizophrenia and mood disorders. Molecular Psychiatry. 2002;7(9):985.
349. ter Heegde F, De Rijk RH, Vinkers CH. The brain mineralocorticoid receptor and stress resilience. Psychoneuroendocrinology. 2015;52:92-110.
350. Gaines R. Scoliosis and kyphosis, review and current concepts. Missouri Medicine. 1980;77(3):124.
351. Moslehi M, Saiiari A, Marashiyan F. Study of the relationship between Kyphosis, anxiety, depression and aggression of high school boy students. Procedia-Social and Behavioral Sciences. 2011;15:1798-801.
352. Beery AK, Kaufer D. Stress, social behavior, and resilience: insights from rodents. Neurobiology of Stress. 2015;1:116-27.
353. Pastel E, Pointud J-C, Martinez A, Lefrançois-Martinez AM. Aldo-keto reductases 1B in adrenal cortex physiology. Frontiers in Endocrinology. 2016;7.
354. Gómez F, Lahmame A, de Kloet R, Armario A. Hypothalamic-pituitary-adrenal response to chronic stress in five inbred rat strains: differential responses are mainly located at the adrenocortical level. Neuroendocrinology. 1996;63(4):327-37.
355. Blazer DG, Kaplan BH. Controversies in community-based psychiatric epidemiology: let the data speak for themselves. Archives of General Psychiatry. 2000;57(3):227-8.
356. Beuschlein F, Heshkovitz L, Klammer S, Shapiro I, Krup M, Weinstein Y. The adrenal X-zone is involved in progesterone inactivation. Experimental and Clinical Endocrinology & Diabetes. 2006;114(S 1):OR2_09.
357. Jorgensen JS, Nilson JH. AR suppresses transcription of the LHβ subunit by interacting with steroidogenic factor-1. Molecular Endocrinology. 2001;15(9):1505-16.
358. Lanzino M, Maris P, Sirianni R, Barone I, Casaburi I, Chimento A, et al. DAX-1, as an androgen-target gene, inhibits aromatase expression: a novel mechanism

blocking estrogen-dependent breast cancer cell proliferation. Cell death & Disease. 2013;4(7):e724.
359. Yang F, Li X, Sharma M, Sasaki CY, Longo DL, Lim B, et al. Linking β-catenin to androgen-signaling pathway. Journal of Biological Chemistry. 2002;277(13):11336-44.
360. Chamorro MN, Schwartz DR, Vonica A, Brivanlou AH, Cho KR, Varmus HE. FGF-20 and DKK1 are transcriptional targets of β-catenin and FGF-20 is implicated in cancer and development. The EMBO journal. 2005;24(1):73-84.
361. Kazanskaya O, Glinka A, del Barco Barrantes I, Stannek P, Niehrs C, Wu W. R-Spondin2 is a secreted activator of Wnt/β-catenin signaling and is required for Xenopus myogenesis. Developmental Cell. 2004;7(4):525-34.
362. Logan CY, Nusse R. The Wnt signaling pathway in development and disease. Annu Rev Cell Dev Biol. 2004;20:781-810.
363. Rainey WE, Saner K, Schimmer BP. Adrenocortical cell lines. Molecular and cellular endocrinology. 2004;228(1):23-38.
364. Dart DA, Waxman J, Aboagye EO, Bevan CL. Visualising androgen receptor activity in male and female mice. PLoS One. 2013;8(8):e71694.
365. Goodman DG. Subcapsular-cell hyperplasia, adrenal, mouse. Endocrine System: Springer; 1996. p. 464-7.
366. Duparc C, Moreau L, Dzib JFG, Boyer H-G, Tetsi Nomigni M, Boutelet I, et al. Mast cell hyperplasia is associated with aldosterone hypersecretion in a subset of aldosterone-producing adenomas. The Journal of Clinical Endocrinology & Metabolism. 2015;100(4):E550-E60.
367. Li L, Dang Q, Xie H, Yang Z, He D, Liang L, et al. Infiltrating mast cells enhance prostate cancer invasion via altering LncRNA-HOTAIR/PRC2-androgen receptor (AR)-MMP9 signals and increased stem/progenitor cell population. Oncotarget. 2015;6(16):14179.
368. Goldstein DS. Adrenal responses to stress. Cellular and Molecular neurobiology. 2010;30(8):1433-40.
369. Juruena MF, Cleare AJ, Papadopoulos AS, Poon L, Lightman S, Pariante CM. Different responses to dexamethasone and prednisolone in the same depressed patients. Psychopharmacology. 2006;189(2):225-35.
370. O'Tuathaigh C, Babovic D, O'Sullivan GJ, Clifford JJ, Tighe O, Croke DT, et al. Phenotypic characterization of spatial cognition and social behavior in mice with 'knockout'of the schizophrenia risk gene neuregulin 1. Neuroscience. 2007;147(1):18-27.
371. Moy S, Nadler J, Perez A, Barbaro R, Johns J, Magnuson T, et al. Sociability and preference for social novelty in five inbred strains: an approach to assess autistic-like behavior in mice. Genes, Brain and Behavior. 2004;3(5):287-302.
372. Porsolt R, Bertin A, Jalfre M. Behavioral despair in mice: a primary screening test for antidepressants. Archives Internationales de Pharmacodynamie et de Thérapie. 1977;229(2):327-36.
373. Frye CA, Edinger KL. Testosterone's metabolism in the hippocampus may mediate its anti-anxiety effects in male rats. Pharmacology Biochemistry and Behavior. 2004;78(3):473-81.
374. Edinger KL, Frye CA. Testosterone's anti-anxiety and analgesic effects may be due in part to actions of its 5α-reduced metabolites in the hippocampus. Psychoneuroendocrinology. 2005;30(5):418-30.

375. Zuloaga DG, Morris JA, Jordan CL, Breedlove SM. Mice with the testicular feminization mutation demonstrate a role for androgen receptors in the regulation of anxiety-related behaviors and the hypothalamic–pituitary–adrenal axis. Hormones and Behavior. 2008;54(5):758-66.
376. Chadman KK, Yang M, Crawley JN. Criteria for validating mouse models of psychiatric diseases. American Journal of Medical Genetics Part B: Neuropsychiatric Genetics. 2009;150(1):1-11.